7-30-2012 12:00 AM

The Role of Transforming Growth Factor Alpha in Osteoarthritis and Skeletal Development

Shirine E. Usmani
The University of Western Ontario

Supervisor
Dr. Frank Beier
The University of Western Ontario

Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

THE ROLE OF TRANSFORMING GROWTH FACTOR ALPHA IN OSTEOARTHRITIS AND SKELETAL DEVELOPMENT

(The role of TGFα in osteoarthritis and skeletal development)

(Thesis format: Integrated Article)

by

Shirine Elizabeth Usmani

Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

Dr. Frank Beier

Supervisory Committee

Dr. Dean Betts (GSR)

Dr. Moshmi Bhattacharya

Dr. Jeffrey Dixon

Examiners

Dr. Tom Drysdale

Dr. David O'Gorman

Dr. Cheryle Seguin

Dr. Rosa Serra

The thesis by

Shirine Elizabeth Usmani

entitled:

The role of transforming growth factor alpha in osteoarthritis and skeletal development

is accepted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Date

Chair of the Thesis Examination Board

Abstract

Osteoarthritis (OA) is the most common joint disease and a major cause of physical disability, however no disease modifying treatments are available. To better understand the molecular mechanisms involved, my colleagues previously performed microarray studies on a surgical rodent model of OA. Transforming growth factor alpha (TGFα) was identified as a novel growth factor involved in cartilage degeneration and subsequent experiments revealed that it induced an OA-like phenotype in articular chondrocytes. These findings lead to the overarching hypothesis for my thesis: TGFα promotes OA.

I began by studying TGFα's interaction with endothelin receptor A (ET(A)R) as this signaling system is known to be involved in both aging and OA. I used *in vitro* cultures to examine whether TGFα-effects were regulated by this pathway and found that TGFα induced expression of ET(A)R at both the gene and protein level. By inhibiting ET(A)R, I was able to partially block some deleterious TGFα effects, however, it was evident that additional downstream targets were involved in TGFα signaling.

To examine the role of TGFα *in vivo*, I used the *Tgfa* null mouse. I first examined bone development as developmental events are often recapitulated in disease. *Tgfa* null mice had a transient growth plate phenotype characterized by an expanded hypertrophic zone and a delay in the transition from cartilage to bone. I also observed a decrease in RANKL and MMP13 gene expression as well as fewer osteoclasts as the cartilage/bone junction. These data suggest that decreased matrix resorption is responsible for the persistence of the hypertrophic zone.

Lastly, I examined post-traumatic and aging OA models. Interestingly, I found that young adult *Tgfa* null mice were protected from developing OA in the traumatic model, however they were not protected with age. These data suggest that TGFα might be more relevant as a therapeutic target in post-traumatic subtypes of OA but not necessarily in idiopathic disease.

Overall, these data demonstrate a novel role for TGFα in both bone development and OA. My developmental and disease data support my hypothesis, as TGFα appears to be a major regulator of cartilage catabolism in both systems.

Keywords

Transforming growth factor alpha, osteoarthritis, articular cartilage, endothelin receptor A, endochondral ossification, osteoclasts, MMP13, Rankl, DMM

Co-Authorship Statement

Chapter 2 is adapted from: Usmani SE, Appleton CT, and Beier F. Transforming growth factor-alpha induces endothelin receptor A expression in osteoarthritis. *J Orthop Res* 30: 1391-1397, 2012. S.E.U. performed most experiments and contributed to study design and manuscript writing. C.T.A. performed the experiments relating to the sugical rat model of osteoarthritis that provided tissues for Figure 2.1. F.B. contributed to study design and editing of the manuscript. All authors read and approved the submitted version of the manuscript.

Chapter 3 is adapted from: Usmani SE, Pest MA, Kim G, Ohora SN, Qin L, and Beier F. Transforming growth factor alpha controls the transition from hypertrophic cartilage to bone during endochondral bone growth. *Bone* 51: 131-141, 2012. S.E.U. performed most experiments and contributed to study design and manuscript writing. M.A.P. assisted with long bone isolations and skeletal preparations. G.K. assited with embryonic dissections and performed blinded measurements. S.N.O helped with breeding of the *Tgfa* null mice as well as P0 dissections. L.Q. is a collaborator who provided experimental advice and assisted with manuscript editing. F.B. contributed to study design and editing of the manuscript. All authors read and approved the submitted version of the manuscript.

Chapter 4 S.E.U. performed most experiments and contributed to study design and manuscript writing. V.U. assisted with scoring of all DMM tissues. I.W. and T.L.H. performed all DMM surgeries. F.B. contributed to study design and editing of the manuscript. These data have not yet been published.

Acknowledgments

I would like to take this opportunity to thank the many people who helped make my graduate student experience such a positive one. First and foremost, I have to thank my supervisor Dr. Frank Beier. Without a doubt you significantly influenced my career path the day you let me volunteer in your lab (I would never have thought I would still be living in London and going to school almost seven years later)! I must thank you for giving me the opportunity to volunteer as a young and eager undergrad and develop a true curiosity and passion for research. I have no doubt that research will always be a part of my career and life plans from now on. I must also thank you for your constant support and patience. Regardless of how many people were in the lab, or where you were in the world, you always made time to answer questions, sit down for meetings, or reply to my emails (which sometimes came at very strange times of the day). What I am probably most grateful for, however, is your genuine concern for your students. While the lab is a primary focus, you have always encouraged your students to travel, gain teaching experience, and most importantly take time off. Your understanding of the 'bigger picture' makes you a wonderful supervisor, and it also makes your students want to work hard to make you proud. For that I thank you!

I must also thank the members of my Advisory Committee (past and present)- Dr. Moshmi Bhattacharya, Dr. Jeff Dixon, Dr. Andy Watson, and Dr. Dean Betts, as well as the members of my MD/PhD Advisory Committee – Dr. Jane Rylett and Dr. Jim Lewis. Your positive feedback and constructive criticisms have really helped to guide me throughout this whole process and most importantly, keep me on track! I feel lucky to have been mentored by such a positive and collaborative group of individuals.

Being a member of the Beier Lab for the past six and a half years, I have had the pleasure of meeting and becoming friends with many wonderful people (late night dissections and cell culture contaminations seem to have a way of bringing people together)! I have to first say thanks to Tom A since he was the first Beier lab member I had the pleasure of working with and learning from. Anita, your kindness and willingness to help others was always much appreciated (especially when I first began in the lab and had no idea what I was doing)! Gouyan, thank you for being a wonderful colleague and I've enjoyed our chats across the lab bench.

Chantal T, Sadia L, and Emily L – thank you for your friendship and the many laughs we've shared together. Sadia and Emily, I will never forget the night you helped me dissect until one in the morning. I was prepared for an awful night, but it actually turned out to be quite the opposite ("We ride together, we die together."). Chantal, I will never forget our experience with Julio the piñata, our 'special' art classes, or your rap video! Thank you for your friendship ladies, and for the many happy memories!

I must also thank the people who helped me out on pretty much a daily basis while I was in the lab. Lauren S – you are the resident expert on many things! I have no idea how you know so much about so much, but thank you for sharing your knowledge with the rest of us! Ryan G (aka big Ryan), I loved having my bench next to the 'man cave' not only because I could easily access a growth plate expert, but for the many entertaining conversations we had. Veronica U – I don't know what I would have done without you! You know that I think of you as my 'life role model', but I just hope you know how serious I am. Your work ethic, contentiousness, and kindness are definitely qualities to strive for. I hope that we can work together again at some point in the future!

I would also like to thank those individuals who collaborated with me and contributed to my research in a meaningful way. Vasek P- You have been such a wonderful help with all of my animal studies! I don't know how know everything would have gotten done without your expertise and skill. Mohit K and Jason R – thank you for your helpful advice and for assisting me with various protocols over the years. I truly appreciate it! Christina F, Gunwoo K and Sara O – Thank you for contributing to my growth plate studies. I know that the dissections and measurement were often tedious, but your help and friendship meant a great deal. Mike P – Thank you so much for the many hours of skeleton cleaning and picture taking that you contributed from the very beginning. You were also the best travel buddy over the years!

I can't accurately describe how much I appreciate Holly D, Dawn B, and Tom C (aka Greek Tom). You are the most wonderful lab technicians, ever! I feel so lucky to have had you all around for help and support. Dawn, I have no idea how you stay so cheery all the time, but I am very thankful that you do. Holly, your honesty and humor are refreshing and have frequently brightened my day. Greek Tom, thank you for teaching me everything from sectioning mouse limbs to making baklava! You are all such talented and wonderful people and I sincerely thank you for your help and friendship over the years.

I must also thank the extended Lower Ground family for their friendship and support. Matt M, Nicole W, and Kim B - things definitely wouldn't have been the same without you! Matt G - thank you for being such a wonderful colleague and roommate over the years! Time has flown by, but I'm so glad that I had you to go through this experience with. I know you will go on to do great things for the world of Science!

Ryan P, I feel so lucky that cartilage brought us together. Whether you are across a lab bench or across an ocean, I know you are there for me once hundred percent and I couldn't ask for anything more than that. I believe that meeting you was a true blessing.

Most importantly, I have to thank my family, as they have provided me with unwavering love and support throughout my life and have always kept me grounded. Uncle Gerry, thank you for always being there to support and love me throughout life's milestones. Your thoughtfulness is unmatched and I am so lucky to have you in my corner. Yasmine, you are the best sister and friend that I could have ever asked for. You know just what to say to motivate me, support me, and make me feel loved. Mom and Dad, thank you for all of the sacrifices you have made for Yasmine and I, and for teaching us the value of hard work. There is no doubt that we are where we are today because of you. You not only encouraged us to follow our dreams and reach our potential, but you did everything in your power to make our dreams a reality. Mom, I want you to know how much your support in this achievement has meant to me. There is no doubt in my mind that this degree belongs just as much to you as it does to me. Finally, I would like to dedicate my thesis to my Grandmother, Elizabeth Voros. She was a wonderful woman who did everything for her family and was an inspiration to us all. My Grandma suffered from osteoarthritis during her later years, and unknowingly influenced the direction of my research career.

Table of Contents

List of Figures

List of Appendices

List of Abbreviations

Acg1	aggrecan gene
ACL	anterior cruciate ligament
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AR	amphiregulin
BMP	bone morphogenetic protein
BMU	basic multicellular unit
BTC	betacellulin
Col2a1	type II collagen gene
COLX	type X collagen
DMM	destabilization of the medial meniscus
DMOAD	disease modifying osteoarthritic drug
E15.5	embryonic day fifteen and a half
Ednra	endothelin receptor A gene
EGF	epidermal growth factor
EGFR	epidermal growth factor
EPGN	epigen
EREG	epiregulin
ET-1	endothelin-1
ET(A)R	endothelin receptor A
ET(B)R	endothelin receptor B
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GPCR	G-protein coupled receptor
HBEGF	heparin-binding EGF-like growth factor

Het	heterozygous
HZ	hypertrophic zone of the growthplate
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
Ihh	Indian hedgehog
IL-1	Interleukin-1
iNOS	inducible nitric oxide synthase
KO	knockout
MAPK	mitogen activated protein kinase
MIG6	mitogen-inducible gene 6
MMP	matrix metalloproteinase
NO	nitric oxide
NF-κB	nuclear factor kappa B
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
OPG	osteoprotegerin
Osx	osterix
P0	post-natal day zero
P21	post-natal day twenty-one
PECAM-1	platelet endothelial cell adhesion molecule-1
PI3K	phosphatidylinositol 3-kinase
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
PZ	proliferative zone of the growth plate
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor kappa B

RANKL	receptor activator of nuclear factor kappa B ligand
ROCK	Rho kinase
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
Runx2	runt-related transcription factor 2
RZ	resting zone of the growth plate
Sox9	sex reversal Y-related high-mobility group box protein
SOC	secondary ossification center
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TRAP	tartrate resistant acid phosphatase
VEGF	vascular endothelial growth factor
WT	wild type

Chapter 1

1 Literature Review

1.1 Skeletal Development

The skeleton distinguishes vertebrate species from invertebrate ones. It is formed from two tissues types: cartilage (produced by chondrocytes) and bone (produced by osteoblasts) (98). Both chondrocytes and osteoblasts are derived from a common mesenchymal precursor referred to as the osteochondroprogenitor. There are two main processes through which bone development occurs: endochondral ossification and intramembranous ossification (59). Both processes begin with the recruitment of mesenchymal precursor cells to the location where the future bone will develop (40). Next, the mesenchymal cells form condensations via adhesion molecules and form the template that will eventually be replaced by bone (40).

1.1.1 Intramembranous Ossification

Intramembranous ossification occurs in several areas of the body including the flat bones of the skull (59). During this process, the condensations of mesenchymal precursor cells differentiate directly into osteoblasts and subsequently produce bone matrix (59). Runx2 and Osx (osterix) are two transcription factors that are absolutely essential for osteoblastogenesis as mice with null mutations for either of these genes lack osteoblasts (74). Most bones in the body do not form directly from osteoblasts however, but rather form through a cartilage intermediate during the process of endochondral ossification.

1.1.2 Endochondral Ossification

Endochondral ossification is the process through which the long bones of the body grow and develop. As opposed to intramembranous ossification, cells within the mesenchymal condensations differentiate into chondrocytes and form a cartilaginous template that will eventually be replaced by bone (59). Chondrogenesis itself is regulated by a number of transcription factors, one of most important being Sox9 (sex reversal Y-related high-mobility group box protein) (57). Sox9 directly regulates a number of genes critical for chondrocyte function including *Col2a1* (type II collagen) and *Agc1* (aggrecan) (57). It is also necessary for the formation of mesenchymal condensations, the expression of *Runx2*, the expression of other *Sox* family members, as well as chondrocyte differentiation and proliferation (4, 57).

Endochondral ossification continues when cells at the periphery of the cartilage template become flattened, producing a boarder of perichondrium (12, 59). The perichondral cells retain the ability to differentiate into either osteoblasts or chondrocytes (61). Next, driven by Sox9, cells in the center of the template begin to proliferate and produce a characteristic matrix composed of type II collagen and aggrecan (12, 59). Eventually cells near the center of the template stop proliferating and undergo hypertrophy (12, 59). This step of terminal differentiation is controlled by several transcription factors including Runx2 (57). The hypertrophic chondrocytes are unique in that they produce a matrix predominantly containing type X collagen and promote vascular invasion through secretion of vascular endothelial growth factor (VEGF) (12, 59). They also signal to adjacent perichondral cells through Indian hedgehog (Ihh), causing them to differentiate into osteoblasts and form the bone collar (12). After they have secreted their matrix,

hypertrophic chondrocytes undergo apoptosis and the blood vessels they attracted provide osteoclasts and osteoblasts precursors that will resorb the hypertrophic matrix and lay down true bone (12, 59, 70). The invading blood vessels require the help of degradative enzymes such as matrix metalloproteinases 13 and 9 (MMP13 and MMP9) to break down the horizontal septa of matrix (70). The vertical septa of cartilage matrix between rows of terminal chondrocytes serve as a scaffold for the formation of this true bone (also known as the primary spongiosa) (12, 59, 70). Over time, this primary bone will be remodeled to secondary trabecular bone (12). Collectively, this area of activity at the center of the cartilage template is referred to as the primary ossification center (59).

1.1.3 Cartilage Growth Plate

During endochondral bone growth, the chondrocytes become highly organized into distinct zones with separate functions (Figure 1.1). The chondrocytes closest to the ends of the bones are small, rounded, and relatively quiescent and are referred to as resting cells (12, 59, 70). Below them, rapidly dividing chondrocytes form columns of flat, coin-shaped proliferating cells and secrete type II collagen and aggrecan (12, 59, 70). When proliferating chondrocytes exit the cell cycle, they first become pre-hypertrophic, then terminally differentiate into hypertrophic chondrocytes. As stated above, these cells secrete a matrix rich in type X collagen that will eventually be resorbed and replaced with true bone (12, 59, 70). Hypertrophic chondrocytes are easily distinguishable histologically due to their significantly increased volume (12). Collectively, this organizational arrangement is referred to as the cartilage growth plate and one growth plate is present on either end of a developing long bone.

Figure 1.1 The zones of the cartilage growth plate. The cartilage growth plate is highly organized with several distinct zones. The resting zone consists of small, round, and relatively quiescent cells near the ends of the bone. Next is the proliferating zone, which consists of stacks of flattened, coined-shaped cells. Once these cells stop proliferating, they enlarge, becoming prehypertrophic before they terminally differentiate into hypertrophic chondrocytes.

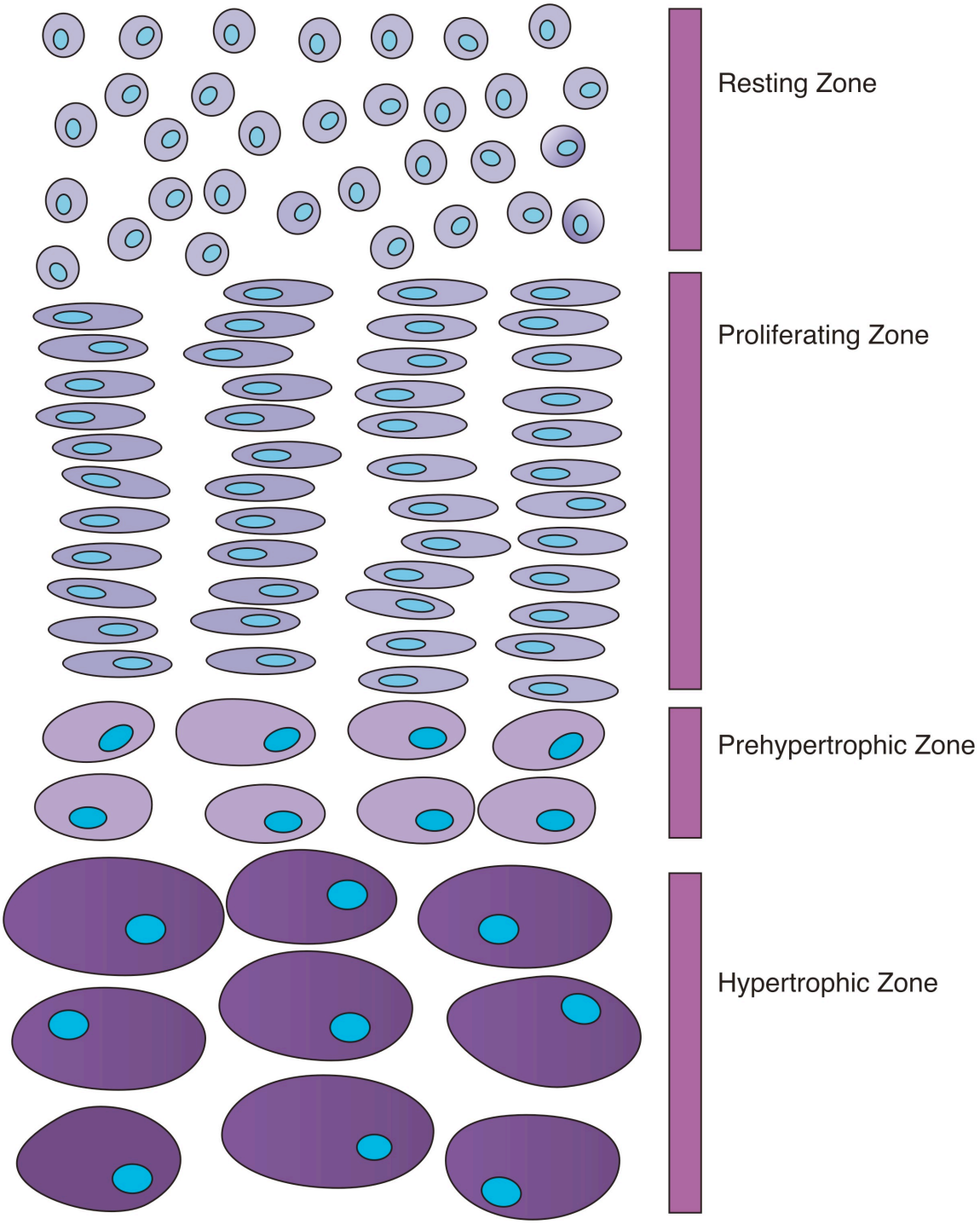


Figure 1.1 The zones of the cartilage growth plate.

1.1.4 PTHrP/Ihh Signaling

Bone development and growth are tightly regulated by both local factors such as fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) as well as systemic factors such as growth hormone, thyroid hormone, and vitamin D (59, 70). The parathyroid hormone related peptide (PTHrP) and Indian hedgehog (Ihh) signaling pathway is an example of the tight control and coordination that is involved within the cartilage growth plate (59). During bone development, perichondral cells at the ends of cartilage template produce PTHrP. The PTHrP receptor (PTH/PTHrP receptor) is a G-protein coupled receptor found on the cell surface of chondrocytes, particularly pre-hypertrophic chondrocytes and those in the lower proliferating zone (12, 60). When activated, the receptor signals to increase SOX9 activity and reduce RUNX2 mRNA and protein, thus maintaining chondrocyte proliferation and inhibition the transition to terminal differentiation (60). Once chondrocytes do stop proliferating, however, they secrete Ihh (Indian hedgehog), which has several roles. Ihh signals to the adjacent perichondral cells and causes them to differentiate into osteoblasts. It also increases the synthesis of PTHrP, to accelerate the differentiation of round proliferating cells to flat, coin-shaped proliferating cells, and increases the rate of proliferation in adjacent chondrocytes (59-61).

1.1.5 Secondary Ossification Centre

As long bones continue to grow, secondary ossification centers form at the proximal and distal ends. These additional ossifications centers form through a similar process as described above: chondrocytes stop proliferating, terminally differentiate, and attract blood vessels that will subsequently lay down new bone (59). The cartilage at the very

ends of bones becomes known as articular cartilage and its main function is to provide smooth movements for diarthrodial joints.

1.1.6 Growth Plate Closure

Longitudinal growth declines as the skeleton reaches maturity. Changes in the growth plate occur, such as decreased proliferation, decreased diameter of the hypertrophic chondrocytes, and decreased cell density (11). In humans, the growth plate closes following puberty and this process is primarily under the control of the sex hormone estrogen (11).

1.2 The Skeleton

The human skeleton is a complex and dynamic structure comprised of over 200 individual elements (51). It provides support and protection for vital organs and bone marrow, as well as attachment sites for muscles thus facilitating movement (39, 51). Bone also has important metabolic functions as it serves as the main reservoir for calcium and phosphate in the body (39). Throughout life, bone constantly remodels and repairs itself thorough the coordinated activities of osteoblasts and osteoclasts, which together are referred to as the basic multicellular unit (BMU) (39, 83).

1.2.1 Bone Matrix

Bone is a porous material containing blood vessels, cells, and a mineralized extracellular matrix. The matrix itself is primarily composed of type I collagen fibers, calcium containing hydroxyapatite crystals, and non-collagenous proteins such as osteocalcin (39). There are two main types of bone found within the skeleton: cortical and trabecular (39). Cortical bone is very dense, has a low turnover rate, and makes up approximately

eighty percent of the skeleton (39). It is located on the outer surfaces of skeletal elements providing strength and protection. Trabecular bone comprises the remaining twenty percent of the skeleton by mass, but it makes up eighty percent of the skeleton by volume (39). Trabecular bone is found within the long bones of the skeleton, and in the inner portions of other bones including the vertebral bodies and the bones of the pelvis (39). In contrast to cortical bone, trabecular bone is less dense and has a high turnover rate, thus making it the primary source for metabolic responses (39).

1.2.2 Bone Remodeling

Bone remodeling occurs constantly throughout life as the skeleton responds to mechanical loads and strains and repairs microfractures (39). The two cell types responsible for this process are the bone-resorbing osteoclasts and the bone-forming osteoblasts, which together are known as the basic multicellular unit (BMU). These two cell types communicate and collaborate to carry out the three major stages of bone remodeling: resorption, reversal, and formation (39, 92).

1.2.3 Osteoclasts

Osteoclasts are derived from hematopoietic stem cells (27, 57). Binding of RANKL (receptor activator of nuclear factor kappa B ligand) to its receptors on pre-osteoclasts causes their activation, fusion, and differentiation to mature multinucleated cells (27, 39). Osteoclasts are equipped with abundant mitochondria and lysosomal enzymes that enable them to carry out their bone-resorbing function (27, 39). The surface of the osteoclast in contact with bone matrix is called the ruffled boarder, and it is along this surface that osteoclasts release their digestive enzymes including tartrate resistant acid phosphatase

(TRAP), cathepsin K, and MMP9 (27, 39). In addition to proteolytic enzymes, osteoclasts release hydrogen ions to acidify the matrix, thus releasing inorganic components (27, 39).

1.2.4 Osteoblasts

Unlike osteoclasts, osteoblasts are derived from mesenchymal stem cells and their differentiation is dependent on the master regulator gene *Runx2* (runt-related transcription factor 2) (57). Osteoblasts are found in clusters along the bone surface where they deposit type I collagen along with other non-collagenous proteins including osteocalcin, osteopontin, osteonection, bone sialoprotein, biglycan, and decorin (27, 39). Collectively, this unmineralized matrix is termed osteoid, and it will eventually become mineralized with the deposition of hydroxyapatite crystals (27, 39). Osteoblasts are also responsible for regulating osteoclast differentiation through the RANKL/OPG (osteoprotegerin) signaling axis (27).

1.2.5 RANKL/OPG Signaling Axis

RANKL is a peptide expressed on the surface of preosteoblasts and stromal cells (39). It is a member of the TNF superfamily and in addition to cell surface expression, it can be cleaved to a soluble form (27, 39, 92). Its receptor, RANK, is located on the surface of osteoclast precursor cells and when activated, initiates osteoclast differentiation and activation (39, 92). RANKL is absolutely required for osteoclastogenesis as mice lacking RANKL fail to produce osteoclasts (75). Cells of the osteoblast lineage also produce a soluble decoy receptor, OPG that binds to RANKL, inhibiting its ability to activate RANK (27, 39, 92). This coupling of osteoclast and osteoblast activity produces an efficient and coordinated process of bone remodeling.

1.3 Articular Cartilage

1.3.1 Cartilage Classification

Cartilage is a type of connective tissue found throughout the body with various roles depending on its location and subtype. There are three major histological classifications of cartilage based on the predominant structural components of the extracellular matrix: elastic, fibro-, and hyaline cartilage (22). Elastic cartilage is located in the epiglottis and eustachian tube, and as the name suggest, contains a high proportion of elastic fibers (22). Fibrocartilage is found in the intervertebral discs of the spine as well as the menisci of the knee joints and has a high proportion of fibrous tissue rich in type I collagen fibers (22, 81). Hyaline cartilage forms the growth plate of long bones and provides a template that will eventually be replaced by bone. Hyaline cartilage is also found at the ends of diarthrodial joints forming articular cartilage, and its predominant matrix component is type II collagen (22, 81).

1.3.2 Articular Cartilage Composition

Articular cartilage is the highly specialized tissue that covers joint surfaces, giving rise to near frictionless, wear-resistant and efficient movements. There are two main components of articular cartilage: the chondrocytes (the cells of articular cartilage) and the extracellular matrix in which they reside. Articular cartilage is relatively acellular compared to other tissues in the body, with chondrocytes comprising only about two percent of the total cartilage volume in adults (81). Articular cartilage is avascular, aneural, and alymphatic, and consequently the chondrocytes themselves are primarily responsible for secreting components of the extracellular matrix and for regulating much of its turnover (3). The vast majority of articular cartilage is comprised of the

extracellular matrix which is rich in type II collagen, proteoglycans such as aggrecan, hyaluronic acid, and cross-linking proteins (3). Articular cartilage is a dynamic tissue as type II collagen provides the main structural network and tensile strength while aggrecan attracts water providing elastic properties (3). Due to the low cell density and lack of blood supply however, articular cartilage is limited in its ability to self-repair.

1.3.3 Zonal Organization

Articular cartilage is designed to absorb and distribute the various stresses experienced during joint loading (55, 81). It can be divided into three major zones (superficial, middle, and deep), each with its own unique cellular distribution and extracellular matrix arrangement (Figure 1.2). The superficial zone is relatively thin, and contains horizontally arranged collagen fibrils in parallel with the articular surface (55). The chondrocytes in the superficial zone are elliptical in shape and are also found parallel to the surface (55). These chondrocytes synthesize and secrete a protein called lubricin, which as the name implies, helps to lubricate and maintain a smooth, frictionless articulating surface (81). In addition, the superficial zone is crucial to the function of articular cartilage as it is able to withstand tensile forces experienced during joint loading (81). In the middle zone, chondrocytes are spherical in shape and are surrounded by randomly distributed collagen fibrils (55). Proteoglycan content increases in the midzone, and continues to increase as one moves deeper within the articular cartilage layers (81). This is important because aggregating proteoglycans attract water molecules and provide articular cartilage with its elastic properties (3). Proteoglycan aggregates form when aggrecan monomers attach to a hyaluronic acid backbone via link protein (55, 81). These

Figure 1.2 The zones of articular cartilage. The function of articular cartilage is dependent on its zonal organization. The superficial zone consists of elongated chondrocytes situated in parallel with the articular surface. Collagen fibrils in this zone are also parallel to the surface. Next is the middle zone, which is characterized by spherical chondrocytes surrounded by more randomly distributed collagen fibrils. Beyond this lays the deep zone containing columns of chondrocytes surrounded by collagen fibrils perpendicular to the surface. The tidemark divides the articular cartilage from the calcified cartilage below.

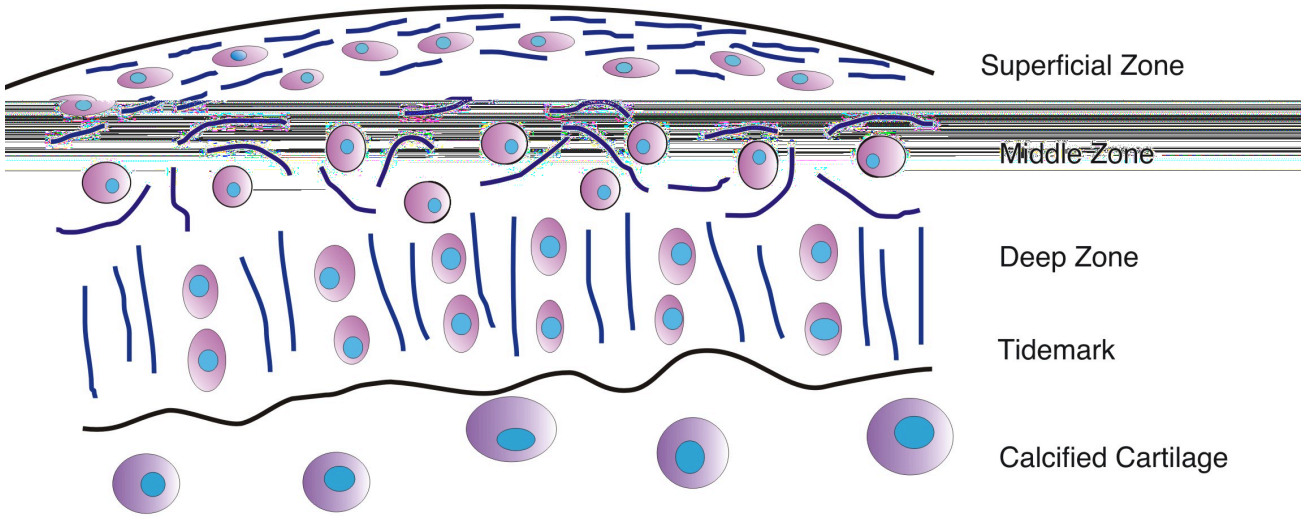


Figure 1.2 The zones of articular cartilage.

monomers are composed of a protein core to which negatively charged glycosaminoglycans (GAGs) such as chondroitin sulfate and keratan sulfate attach (55). The negatively charged molecules attract and bind intercellular water which can be essentially ‘squished out’ under compressive forces, and drawn back in after the joint has been unloaded (3). Lastly, the deep zone contains large chondrocytes arranged in columns and collagen fibrils here are found perpendicular to the articular surface (55). Between the deep zone and the subchondral bone exists a layer of calcified cartilage which contains very large chondrocytes that behave similarly to the hypertrophic chondrocytes of the cartilage growth plate (81). These chondrocytes secrete type X collagen that can subsequently be mineralized. The ‘tidemark’ is a histologically visible line demarcating the articular cartilage from the underlying calcified cartilage. The calcified cartilage is believed to provide a transition zone from cartilage to subchondral bone (81).

1.3.4 Regional Organization

In addition to the zonal organization of the articular cartilage, the extracellular matrix is also described based on its proximity to the chondrocytes themselves (3). The matrix directly adjacent to the chondrocytes is called the pericellular matrix and is rich in type VI collagen and proteoglycans (36). The interterritorial matrix occupies the largest proportion of articular cartilage and refers to the matrix farthest from the chondrocytes (36). As mentioned, this proportion of the matrix is comprised primarily of type II collagen, aggrecan, and water and allows the cartilage to deal with compressive forces. The cartilage between the pericellular and interterritorial matrix is referred to as the territorial matrix, but currently has no specific characterization (3).

1.3.5 Cell-Matrix Interactions

Chondrocytes are in physical contact with their pericellular environment through cell surface receptors such as CD44 and integrins that directly bind matrix molecules (36). These cell surface receptors provide a direct means through which chondrocytes can interact with and to respond to their extracellular environment and mechanical stimuli. In addition, articular chondrocytes express stretch-activated calcium channels that respond to mechanical stimuli by modulating intracellular calcium waves. These fluxes in calcium subsequently cause the release of growth factors and cytokines that can then signal in an autocrine and/or paracrine manner (2, 23). Finally, the chondrocyte cytoskeleton appears to play an important role in mediating cellular events. In all cell types, the cytoskeleton serves as the load-bearing architecture of the cell and can be altered by various forces (5). Several studies have indicated that disruption of the actin cytoskeleton causes chondrocyte de-differentiation to a fibroblastic-like phenotype (16).

1.3.6 Extracellular Matrix Metabolism

Articular chondrocytes exist in an avascular, relatively hypoxic environment (33, 35). Consequently, they are relatively metabolically inactive and have a low regenerative capacity. Glucose is their main energy source and enters the cells via facilitated transport (33). Glucose also serves as a precursor for the intracellular production of glycosaminoglycans (GAGs) (33). Adult articular cartilage has a low turn over rate with the half-life of collagen being about one hundred years and the half-life of aggrecan core protein fractions ranging from three to twenty-four years (33). Matrix metabolism may however be accelerated in the pericellular zone and may also vary in disease states. Anabolic factors including transforming growth factor beta (TGFβ) and insulin-like

growth factor-1 (IGF-1) stimulate extracellular matrix (ECM) synthesis by chondrocytes (87). Conversely, inflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) stimulate catabolic factors (i.e. matrix metalloproteinases) and are thus associated with matrix destruction (87). In healthy cartilage there is an overall balance between matrix synthesis and degradation (3). In the disease state of osteoarthritis (OA) however, the balance is shifted in favour of degradation leading to subsequent joint dysfunction.

1.4 Osteoarthritis

Osteoarthritis (OA) is a chronic degenerative joint disease characterized by joint pain, stiffness, and eventual loss of function (93). It is the most common joint disease worldwide and the leading cause of physical disability in industrialized nations (58, 86). It has been reported that one in four people in their sixties and more than half of all people over the age of eighty demonstrate radiographic signs of OA (24). Furthermore, sixty-seven percent of women and fifty-five percent of men over the age of fifty-five have OA of the hand (14). While advanced age is a significant risk factor for the development of the disease, OA can be seen in all age groups (17). Individuals living with OA experience a substantial decline in their health-related quality of life compared to individuals without the disease (85, 97). They suffer most dramatically physically, but also mentally, and socially (97) . Furthermore, unlike rheumatoid arthritis, no disease modifying drugs are available for OA. Therefore, most treatments are long term and are limited to managing symptoms (102). The immense costs associated with OA are both direct (medications, hospitalizations) and indirect (loss of work and productivity) and place a significant burden on our health care system and economy (15, 100). Due to the

prevalence of OA and the disability caused by it, OA poses significant socioeconomic challenges.

1.4.1 Etiology and Risk Factors

While OA can present in any joint in the body, it is most commonly found in the weight bearing joints of the knees and hips as well as in the small joints of the hands (in particular the distal interphalangeal joints) and the spine (17, 93). Although the exact etiology of OA remains elusive, it appears to be multifactorial in nature with many identifiable risk factors. For example, there is good evidence to show that in addition to increasing age, female gender, obesity, joint injury, joint malalignment, and occupations involving repetitive joint loading all contribute the development of OA (17, 104). As well, genetics play a clear role (86, 96). Twin studies have reveal concordance rates of 60% for hip OA and 39% for knee OA based on radiological findings in identical female twins (96). These data are independent of environmental factors. Several gene mutations are associated with OA, some of which encode articular cartilage matrix proteins including type II collagen (86). Other disease associated genes are expressed in bone, skeletal muscle, the cartilage growth plate, or in multiple synovial joint tissues (86). In reality, it is likely that genetic factors give rise to a predisposition for OA but that the mechanical and other risk factors also contribute to disease onset and progression.

1.4.2 Classification

OA can broadly be classified into two main groups: 1) primary or idiopathic and 2) secondary or post-traumatic (17). Primary OA refers to a disease without a definite etiologic cause. It is possible, however, that individuals have an unknown genetic predisposition. Primary OA is rarely found in individuals under the age of 40 (17).

Secondary OA, on the other hand, refers to a disease state in which there is a definite event or abnormality leading to joint degeneration. For example, joint injuries (i.e. ligament or meniscal tears), intra-articular fractures, and congenital abnormalities (i.e. hip dysplasia) can all result in joint degeneration (6, 17, 104). Unlike primary OA, secondary OA is often found in younger individuals. Regardless of the classification, both forms of OA are complex, multivariable, and affect the entire joint. While rates of progression may be variable, subsequent disease sequelae are often indistinguishable and involve joint pain, stiffness, and loss of function (17).

1.4.3 A Disease of the Synovial Joint

While degeneration of articular cartilage is the hallmark of OA, it is important to remember that the disease actually affects the entire synovial joint. In addition to progressive cartilage damage, an osteoarthritic joint often involves subchondral bone sclerosis, synovitis (inflammation of the synovial membrane), and osteophyte formation (bony outgrowths at the joint margin) (3, 47) (Figure 1.3).

1.4.3.1 Articular Cartilage

While articular cartilage degeneration is the defining feature of OA, a number of more subtle changes also occur within the extracellular matrix and the chondrocytes themselves. Cartilage degeneration often begins with fibrillations at the articular surface which eventually penetrate into the deeper zones (80). Another early sign of osteoarthritis is chondrocyte proliferation resulting in clustering of cells (34, 87). Early proliferation may be a consequence of early cartilage damage that subsequently allows mitogenic factors from other tissues such as the synovium to reach the chondrocytes (86). Chondrocytes may also de-differentiate and lose their characteristic phenotype of type II

collagen and aggrecan expression (78, 87). Other chondrocyte changes that have been observed are hypertrophy, apoptosis, and autophagy although their actual contribution to OA pathology is not quite clear (13, 87). These processes, like osteophyte formation, are reminiscent of stages in endochondral bone development.

1.4.3.2 Synovium

The synovial membrane lines the joint capsule and contains an inner layer of metabolically active cells called synoviocytes (3). The synoviocytes help to nourish the avascular articular cartilage, remove cartilage breakdown products from joint space, and produce synovial fluid proteins including lubricin and hyaluronic acid (3, 91). The proteins help to lubricate the joint and reduce friction, thus protecting the articular cartilage (91). Histological changes observed in OA can be seen in both early and late stages of the disease and include synovial cell hyperplasia, inflammation (involving the invasion of leukocytes), and capsular fibrosis (58, 91). The synoviocytes can also contribute directly to cartilage degeneration via production of inflammatory cytokines and catabolic enzymes (91).

Figure 1.3 A schematic representation of a healthy and osteoarthritic joint. Osteoarthritis (OA) is a disease that affects the entire joint. Components of the synovial joint include articular cartilage (blue), the joint capsule (light pink), the synovium (lining the joint capsule), and the subchondral bone (areas directly beneath the articular cartilage). Features of OA are depicted on the right side of the joint and include degradation of articular cartilage, inflammation of the synovium, osteophyte formation, and subchondral bone sclerosis (dark grey).

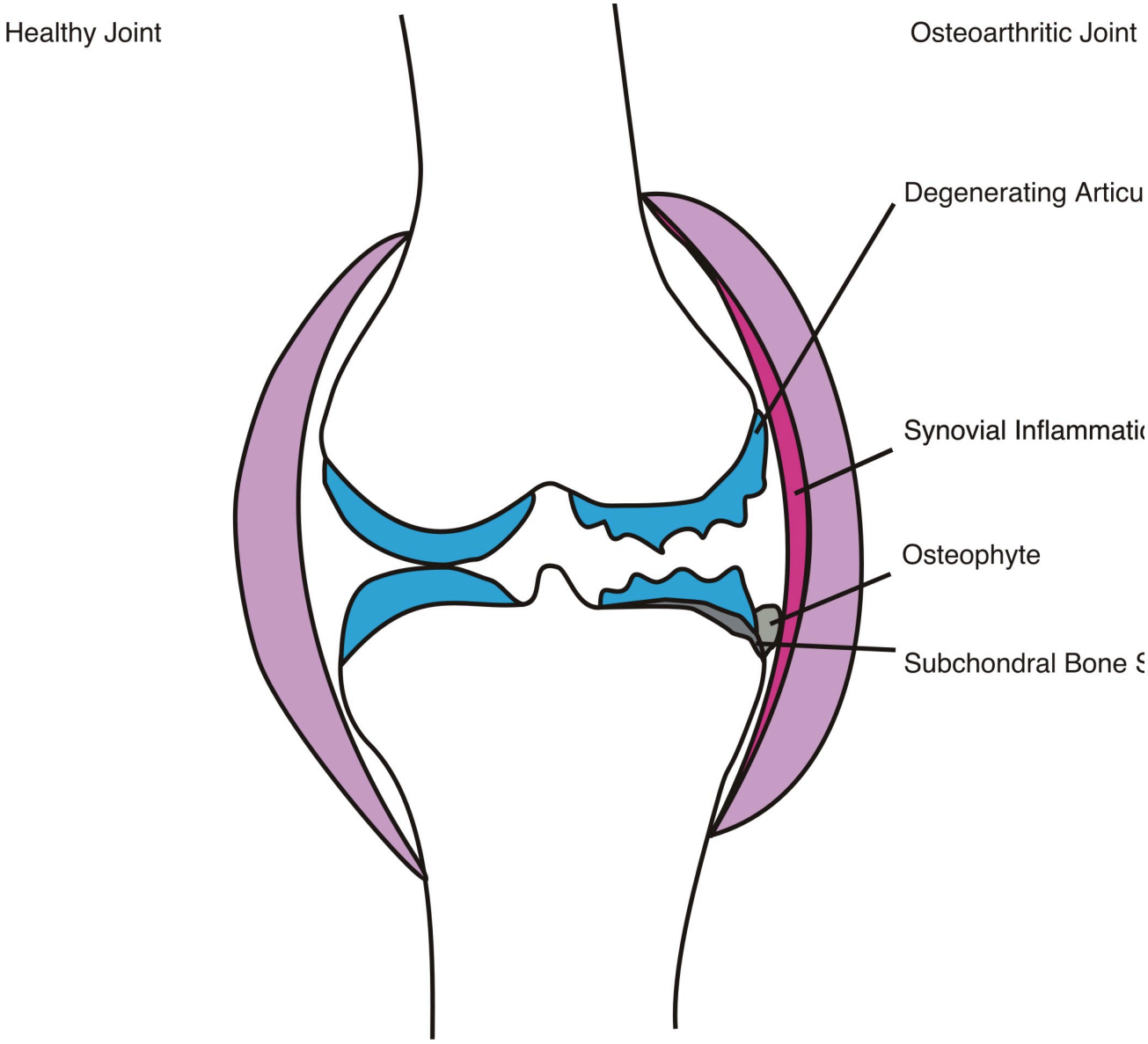


Figure 1.3 A schematic representation of a healthy and osteoarthritic joint.

1.4.3.3 Subchondral Bone

OA is associated with increased subchondral bone remodeling and thickness, however there is uncertainty as to whether this occurs in response to the disease process or whether this is perhaps a causative factor (21, 65). Osteoarthritic subchondral bone is associated with increased osteoblast activity and increased osteoid deposition (64). The increase in bone volume however, is associated with abnormal collagen fibrils and decreased mineralization (65). Animal studies have indicated that increased bone turnover and subsequent subchondral bone thickening are predictive of OA development, thus suggesting that bone changes precede cartilage damage (19). In addition to these subchondral bone changes, reactivation of the secondary ossification centre causes advancement of the cartilage tidemark in OA, effectively thinning the articular cartilage (19, 21). Together subchondral bone sclerosis and the advancing tidemark alter the mechanics of the joint and may potentially exacerbate cartilage damage (19, 21).

1.4.3.4 Osteophytes

Osteophytes are yet another bone-related abnormality found in osteoarthritis. These bony outgrowths form through an endochondral process and may be an attempt to restore stability to a degenerating joint (80, 87). In actuality, however, they may interfere with joint function and mobility. The origin of the osteophytes is uncertain, but they may be derived from undifferentiated cells of the perichondrium (87). The stages of osteophyte development (differentiation, proliferation, hypertrophy) are similar to those observed during endochondral bone growth (87).

1.4.4 Diagnosis

Often patients with OA present to their physicians complaining of joint pain as this is the most prevalent symptom of the disease (93). The diagnosis of OA can be made clinically with a medical history and physical exam (44, 93). No specific tests are required. X-ray is a simple imaging technique that can be used to confirm the diagnosis and reveal the cardinal signs of OA: asymmetric joint space narrowing, the presence of osteophytes (bony outgrowths), subchondral bone sclerosis, and subchondral cysts (44). The joint space narrowing represents loss of articular cartilage at the ends of bones, thus reducing the space between bones. It is important to note that clinical and radiographic findings do not always match and thus lack of radiographic evidence should not exclude the diagnosis of OA (42, 44). Furthermore, if one has radiographic changes consistent with OA but does not experience any symptoms, a diagnosis is not warranted. The limitation of these diagnoses is that patients are often identified very late in the disease course.

1.4.5 Treatment

Currently there is no cure for OA and unlike rheumatoid arthritis, no disease modifying drugs are available to alter the course of the disease. Consequently, treatment options are limited to managing symptoms such as pain and reduced joint function. Treatment options can be divided into broad categories including non-pharmacologic, pharmacologic, complementary and alternative, and surgical (93). Non-pharmacologic interventions include patient education, physiotherapy, weight loss, the use of assistive devices, and activity modification (44, 102). Pharmacologic treatments are targeted at reducing joint pain and include the use of acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular corticosteroid injections, and if necessary

opioid analgesics (93, 102). There has been inconsistent evidence for the use of complementary and alternative treatments such as glucosamine and/or chondroitin sulfate and accupuncture. Thus these treatments may be recommended, but should be discontinued if no relief is experienced (102). Lastly, several surgical options are available. Joint replacement is recommended if other treatments fail to provide symptomatic relief or maintain joint function (102). Osteotomies (surgical alteration of the alignment of the joint) may also be considered in young patients with clear joint malalignment that will likely progress to OA (102).

1.4.6 Natural History

The natural progression of OA varies from person to person with some individuals progressing rapidly and others maintaining a relative stable disease state (30, 31). Several risk factors have been identified for the progression of joint dysfunction including advanced age and obesity (30, 31). For the knee joint in particular, malalignment of the tibiofemoral joint (i.e. varus or valgus alignment) as well as meniscal injuries have been correlated with structural disease progression (30). To complicate matters however, studies have demonstrated that symptomatic OA (joint pain and dysfunction) does not necessarily correlate with structural OA changes found by imaging (42).

1.4.7 The Future of OA

The incidence of OA coupled with the price required to manage the disease has placed a serious strain on our health care system and economy. As the average life expectancy increases and while risk factors such as obesity remain prevalent among our population, the incidence of OA is only expected to increase in the coming years (58). Furthermore, studies have recently shown that in addition to morbidity, OA may increase mortality,

potentially due to lack physical activity secondary to joint dysfunction and the sequelae from long term use of medications, particularly NSAIDs (45). It is for these reasons that a deeper understanding of OA pathology is urgently needed in order to identify effective treatment options and ideally find ways to halt and even reverse disease progression.

1.5 Molecular Mechanisms of Cartilage Degradation

Articular cartilage degeneration is the hallmark of osteoarthritis (OA). Unlike the classic inflammatory disease rheumatoid arthritis (RA), which involves systemic inflammation and symmetric joint damage, the cartilage loss seen in OA is usually asymmetric. Cartilage degeneration can be caused by an increase in catabolic factors, by a decrease in anabolic factors, or by a combination of both. Many signaling molecules play a role in cartilage metabolism including cytokines, growth factors, intracellular mediators, transcription factors, and enzymes (78). Here we will highlight some of the best-studied molecules in OA.

1.5.1 Catabolic Factors

The inflammatory mediators TNFα and IL-1β are two predominant catabolic cytokines involved in the initiation and progression of cartilage degeneration (33, 50). While inflammation definitely plays a role, OA is not considered to be a classical inflammatory disease like RA as it lacks systemic inflammatory markers and the presence of neutrophils in the synovial fluid (33). Synovitis (inflammation of the synovium) is present in osteoarthritis however, as is cartilage inflammation at the molecular level (50, 69). TNFα, IL-1β and their specific receptors are upregulated in OA chondrocytes (43). In osteoarthritis, elevated levels of these cytokines have been observed in a number of joint tissues including the synovial membrane and fluid, subchondral bone, and cartilage

(50). TNFα and IL-1β have anti-anabolic and catabolic effects as they have been shown to reduce type II collagen, proteoglycan, and link protein synthesis and increase a number of catabolic enzymes (50). They also stimulate the production of prostaglandins, nitric oxide (NO) via inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines including IL-6, IL-17, IL-18 (33, 50).

Matrix metalloproteinases (MMPs) are the main catabolic enzymes involved in the degradation of the articular cartilage matrix during the disease state of OA. In healthy cartilage, these enzymes are synthesized by chondrocytes at a low rate as part of normal cartilage maintenance and turnover (43). These enzymes are secreted from the cell into the matrix in a pro-enzyme form, where they are eventually cleaved to their active form by other MMPs or plasmin (87). MMP activity is further regulated by tissue inhibitors of MMPs (TIMPs) whose expression decreases in OA, thus promoting MMP activity (86). MMP13 (collagenase-3) is likely the most important enzyme in OA as it is capable of degrading type II collagen fibrils with the greatest efficiency (43, 87). There are however twenty-three MMP family members, and several including MMP1 (collagenase-1), MMP3 (stromelysin), and MMP8 (collagenase-2) have been implicated in cartilage injury and OA (35, 43, 87).

In addition to MMPs, aggrecanases (enzymes that digest the proteoglycan aggrecan) are critical enzymes involved in OA. In particular, two members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family, namely ADAMTS-4 and ADAMTS-5, are expressed by chondrocytes in osteoarthritis and ADAMTS-4 is synthesized in response to TNFα and IL-1β (46, 78).

In addition to the cytokines and growth factors mentioned above, a number of other signaling molecules have been shown to regulate ECM turnover. A great amount of focus has shifted to the catabolic role of nitric oxide (NO) in OA pathology (89, 95). NO and iNOS are increased in arthritic chondrocytes, and NO itself has been shown to decrease type II collagen and proteoglycan synthesis and increase the expression of catabolic enzymes (1). NO can also induce chondrocyte apoptosis and positively regulate the NF-kB (nuclear factor kappa B) pathway. NF-kB is a transcription factor that mediates the expression of other proinflammatory cytokines (78).

1.5.2 Anabolic Factors

TGFβ is one of the most important anabolic factors in cartilage. It signals through TGFβ receptors and downstream Smad activation to increase type II collagen and proteoglycan synthesis and to decrease catabolic enzyme production (43, 78). It also counteracts the anti-anabolic and catabolic effects of IL-1β (43, 78). In aging and arthritic chondrocytes however, the cell's ability to respond to TGFβ is diminished, thus blunting its anabolic potential (78).

Bone morphogenetic proteins (BMPs) are also members of the TGFβ superfamily and activate downstream Smad signaling (43). Several BMPs are involved in articular cartilage homeostasis. BMP-2 is upregulated in arthritic chondrocytes, possibly in an attempt to maintain matrix integrity (78). BMP-7 which has been shown to delay OA progression in *in vivo* disease models, on the other hand, is downregulated (78).

IGF-1 signals through the IGF-1 tyrosine kinase receptor to stimulate matrix secretion, oppose matrix degradation, and promote chondrocyte survival (33, 43). Interestingly,

IGF-1 gene expression is increased in osteoarthritic chondrocytes, however its ability to signal is blunted by the presence of inhibitory IGF binding proteins (33, 78). Lastly, fibroblast growth factors (FGFs) have a more complicated role in the regulation of articular cartilage metabolism. Studies have shown that FGF-2 (also known as basic FGF) is released from the cartilage matrix under mechanical stress and appears to have a mitogenic effect on chondrocytes (33, 78). This mitogenic effect might be more pathologic than anabolic as increased proliferation of chondrocytes is often associated with a loss of chondrocyte phenotype (78). FGF-18 in contrast, has a more straightforward role stimulating matrix synthesis in articular chondrocytes (78).

1.6 Signaling Pathways in Osteoarthritis

1.6.1 Transforming growth factor alpha

Our laboratory recently identified transforming growth factor alpha (TGFα) as a novel growth factor involved in OA from microarray studies performed on degenerating cartilage in surgical rodent model of the disease (7). TGFα was first discovered in conditioned media from murine sarcoma virus-transformed fibroblastic cells (26). It was discovered at the same time as TGFβ and thus the two molecules have similar nomenclature despite being structurally unrelated (18, 26, 82). Due to the nature of their discovery, TGFα and TGFβ were originally called sarcoma growth factors, but were later more appropriately renamed transforming growth factors (82). TGFα itself is a member of the epidermal growth factor (EGF) family and is known to play key roles in normal growth and development. In particular, TGFα is known to be involved in cell proliferation, migration, and differentiation (67). A variety of embryonic and adult tissues express TGFα including the brain, skin, gastric mucosa, colon, liver and kidney (62).

Furthermore, various tumors and immune cells such as eosinophils, monocytes, macrophages, and neutrophils express TGFα (20, 67, 71, 99).

To better understand the role of TGFα *in vivo*, several mutant mouse models have been studied. Transgenic mice overexpressing *Tgfa* under control of metallothionein promoter developed epithelial hyperplasia, pancreatic metaplasia, hepatocarincoma as well as mammary adenocarcinoma (48, 88). *Tgfa* null mice were also studied and main phenotypic changes reported include wavy fur due to unorganized hair follicle arrangement, curly whiskers, precocious eye-opening, and corneal inflammation (73). Surprisingly, newborn mice appeared to undergo normal post-natal development despite altered TGFα expression (48, 73, 88).

Derived from its 160 amino acid transmembrane precursor (pro-TGFα), the 50 amino acid length TGFα must be proteolytically cleaved to its mature, soluble form (28, 68). Both the membrane bound and soluble forms contain the characteristic EGF-like domain that can bind to the EGF receptor (EGFR) and thereby signal in a juxtacrine or paracrine/autocrine fashion, respectively (25, 62, 67).

1.6.2 Epidermal Growth Factor Receptor Family and Ligands

The epidermal growth factor receptor is a receptor tyrosine kinase (RTK). As an RTK, EGFR binding results in receptor dimerization and activation of the cytoplasmic kinase domains (67, 90). Once activated, EGFR can then stimulate a variety of downstream signaling pathways including the ras/raf/mitogen activated kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, phospholipase C (PLC), and Rho GTPase

pathways (38, 67, 90). The physiological consequences of EGFR activation are highly ligand-and cell type-specific (38, 67, 94).

1.6.3 EGFR Signaling and Osteoarthritis

Growing evidence supports the involvement of EGFR signaling in arthritic diseases. Both RA and OA patients express a variety of cytokines and growth factors in their synovial membranes including IL-1β, TNF-α, and EGF (29). RA patients express higher levels of these cytokines, highlighting quantitative differences in these joint diseases (29). Additional studies have detected TGFα in the synovial fluid and synovial membranes of arthritic patients, and again these levels were higher in those with RA compared to OA (41, 63). Furthermore, TGFα has been detected in control synovium and synovial fluid, suggesting a normal physiological role (41).

The most convincing evidence for the involvement of EGFR signaling in OA pathogenesis comes from recent studies involving mutations in mitogen-inducible gene 6 (MIG6) (105). MIG6 (also known as RALT or Gene 33) is a cytoplasmic protein that negatively regulates EGFR signaling through several mechanisms, including the inhibition of receptor kinase domains of ERBB1/EGFR and ERBB2 and receptor internalization and degradation (32, 37, 101, 103). Homozygous null mutants of the *Mig6* allele were found to develop early degenerative disease in multiple joints including the knee, ankle, and temporomandibular joints (49, 105). The *Mig6* null joints had classical arthritic features such as loss of proteoglycan content, degradation of articular cartilage, formation of subchondral cysts, synovial hyperplasia, osteophyte formation, and abnormal calcification (49, 105). These studies suggest that increased EGFR signaling is sufficient to trigger spontaneous disease progression *in vivo*.

In vitro studies have tried to explain the consequences of EGFR signaling in articular cartilage itself. When treated with exogenous EGF, primary rat articular chondrocytes show a loss of chondrocyte phenotype through altered chondrocyte morphology as well as decreased aggrecan and type II collagen gene expression (56).

Our own *in vitro* studies have shown similar effects with TGFα treatment (8). In primary articular chondrocyte cultures, TGFα decreased expression of the anabolic genes aggrecan (*Agc1*) and type II collagen (*Col2*) while it increased gene expression of the catabolic factors MMP13 (*Mmp13*) and TNFα (*Tnfa*) (7). In addition, TGFα altered chondrocyte morphology and induced stress fiber formation (7). Furthermore, articular cartilage organ cultures treated with TGFα expressed more MMP13 than controls and also displayed other OA-like characteristics such as chondrocyte clustering (8). These data indicate that TGFα treatment encourages OA-like phenotypic changes in chondrocytes. Our collaborators also found that a subset of human OA patients expressed higher than normal TGFα mRNA levels, thus establishing an important correlation between our animal model and the human disease state (7).

After identifying the pathologic effects of TGFα in cartilage, the next logical step was to elucidate its signaling pathways. We used pharmacological inhibitors to block downstream EGFR pathways and found that the RhoA/ROCK pathway and the MEK/ERK branch of the MAPK pathway mediate many TGFα effects (9). Specifically, RhoA/ROCK inhibition blocked TGFα-induced morphologic changes to chondrocytes, MEK/ERK inhibition blocked the down regulation of anabolic genes, and inhibition of both pathways decreased type II collagen cleavage by MMPs (9).

1.6.4 Endothelin Receptor and Osteoarthritis

EGFR signaling is known to overlap and interact with many other signaling systems, including G-protein coupled receptors (GPCRs) (38). Recently, much effort has gone into studying the interactions between EGFR and the GPCR endothelin receptor A (ET(A)R) in cancer cells (10, 66). In articular chondrocytes specifically, it has been observed that EGF induces ET(A)R expression as well as that of its ligand, endothelin-1 (ET-1) (76, 77). Our interest in interactions between these two signaling systems emerged firstly from the fact that like TGFα, ET(A)R gene expression was upregulated in our rodent model of OA (7), and secondly from the body of research implicating both systems in OA disease progression.

ET(A)R and its twenty-one amino acid peptide ligand endothelin-1 (ET-1) are well known for their vasoconstrictive effects on vascular smooth muscle cells as well as their mitogenic effects in cancer cells (66, 79). ET(A)R is a GPCR coupled to the pertussis toxin-insensitive G-protein, Gq (66). Binding of ET-1 activates Gq which in turn stimulates several downstream pathways including the ras/raf/MAPK, PI3K/Akt, and PLC pathways (10, 66). ET-1 is expressed in many cell types and contributes to normal cellular processes such as vasomotor tone, proliferation, and development (66).

There is a great deal of evidence to suggest that both ET(A)R and its ligand play important roles in chondrocyte physiology and pathology. Articular chondrocytes were first shown to produce and release significant quantities of endothelin in 1997 (53). Further studies using rodent articular chondrocytes showed that ET-1 production and ET(A)R density increased with age (52, 77). This trend is significant because age is the number one risk factor associated with OA (17). Prolonged exposure to ET-1 has also

been shown to inhibit proteoglycan and collagen synthesis in rat articular chondrocytes (54). These data are complemented by reports that indicate that ET-1 increases expression of the matrix metalloproteinases MMP1 and MMP13 in human osteoarthritic chondrocytes (72, 84). Human osteoarthritic chondrocytes also respond to ET-1 with increased expression of inducible nitric oxide synthase (iNOS, one enzyme responsible for NO production), as well as elevated NO production (72). Overall, endothelin signaling appears to discourage matrix synthesis and promote degeneration in articular cartilage.

1.7 Overall Objectives and Hypotheses:

While OA affects the entire joint, the hallmark of this disease is the degeneration of articular cartilage. My colleagues have previously identified transforming growth factor alpha (TGFα) as a novel growth factor involved in cartilage degeneration. *In vitro* experiments showed that TGFα had profound effects on the chondrocyte phenotype, reducing anabolic factor expression and increasing catabolic factor expression. Based on these data, the overarching hypothesis for my thesis is that TGFα promotes OA.

1.7.1 Objective #1

To determine the relationship between TGFα and endothelin receptor A (ET(A)R) signaling in articular cartilage.

1.7.1.1 Rationale #1

ET(A)R signaling and TGFα appear to have similar effects on articular chondrocytes. Since both TGFα and ET(A)R are upregulated in the surgical rodent model of OA, the potential link between TGFα and ET(A)R signaling deserves further investigation.

1.7.1.2 Hypothesis #1

TGFα will induce ET(A)R expression, and inhibition of ET(A)R will block TGFα–induced effects in articular cartilage.

1.7.2 Objective #2

To characterize the role of TGFα in skeletal development.

1.7.2.1 Rationale #2

TGFα and its receptor appear to be important mediators of bone development and physiology. However, the role of TGFα in endochondral ossification and growth plate physiology has not yet been defined. I intend to study the role of TGFα in endochondral ossification and characterize the growth plate phenotype of *Tgfa* null mice. Furthermore, since developmental events appear to be recapitulated in OA, this study may provide further insight into the role of TGFα in the disease state.

1.7.2.2 Hypothesis #2

Tgfa null mice will experience delayed endochondral ossification.

Objective #3

To determine the role of TGFα in osteoarthritis progression *in vivo*.

1.7.2.3 Rationale #3

My lab has studied the effects of exogenous TGFα using articular cartilage cell culture and organ culture models. To determine whether these findings are relevant in the *in vivo* disease state, I will evaluate the role of TGFα in both a surgical and spontaneous mouse model of OA.

1.7.2.4 Hypothesis #3

Tgfa null mice will experience delayed OA progression compared to control littermates in both a surgical and an aging model of disease.

1.8 References

1. **Abramson SB.** Osteoarthritis and nitric oxide. *Osteoarthritis Cartilage* 16 Suppl 2: S15-20, 2008.

2. **Aigner T, Kurz B, Fukui N, and Sandell L.** Roles of chondrocytes in the pathogenesis of osteoarthritis. *Curr Opin Rheumatol* 14: 578-584, 2002.

3. **Aigner T, Sachse A, Gebhard PM, and Roach HI.** Osteoarthritis: pathobiology-targets and ways for therapeutic intervention. *Adv Drug Deliv Rev* 58: 128-149, 2006.

4. **Akiyama H, Chaboissier MC, Martin JF, Schedl A, and de Crombrugghe B.** The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev* 16: 2813-2828, 2002.

5. **Alenghat FJ and Ingber DE.** Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins. *Sci STKE* 2002: pe6, 2002.

6. **Anderson DD, Chubinskaya S, Guilak F, Martin JA, Oegema TR, Olson SA, and Buckwalter JA.** Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention. *J Orthop Res* 29: 802-809, 2011.

7. **Appleton CT, McErlain DD, Henry JL, Holdsworth DW, and Beier F.** Molecular and histological analysis of a new rat model of experimental knee osteoarthritis. *Ann N Y Acad Sci* 1117: 165-174, 2007.

8. **Appleton CT, Usmani SE, Bernier SM, Aigner T, and Beier F.** Transforming growth factor alpha suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. *Arthritis Rheum* 56: 3693-3705, 2007.

9. **Appleton CT, Usmani SE, Mort JS, and Beier F.** Rho/ROCK and MEK/ERK activation by transforming growth factor-alpha induces articular cartilage degradation. *Lab Invest* 90: 20-30, 2010.

10. **Bagnato A and Rosano L.** The endothelin axis in cancer. *Int J Biochem Cell Biol* 40: 1443-1451, 2008.

11. **Ballock RT and O'Keefe RJ.** Physiology and pathophysiology of the growth plate. *Birth Defects Res C Embryo Today* 69: 123-143, 2003.

12. **Ballock RT and O'Keefe RJ.** The biology of the growth plate. *J Bone Joint Surg Am* 85-A: 715-726, 2003.

13. **Bertrand J, Cromme C, Umlauf D, Frank S, and Pap T.** Molecular mechanisms of cartilage remodelling in osteoarthritis. *Int J Biochem Cell Biol* 42: 1594-1601, 2010.

14. **Bijlsma JW, Berenbaum F, and Lafeber FP.** Osteoarthritis: an update with relevance for clinical practice. *Lancet* 377: 2115-2126, 2011.

15. **Bitton R.** The economic burden of osteoarthritis. *Am J Manag Care* 15: S230-235, 2009.

16. **Blain EJ.** Involvement of the cytoskeletal elements in articular cartilage homeostasis and pathology. *Int J Exp Pathol* 90: 1-15, 2009.

17. **Buckwalter JA and Martin JA.** Osteoarthritis. *Adv Drug Deliv Rev* 58: 150-167, 2006.

18. **Burgess AW.** Epidermal growth factor and transforming growth factor alpha. *Br Med Bull* 45: 401-424, 1989.

19. **Burr DB.** Increased biological activity of subchondral mineralized tissues underlies the progressive deterioration of articular cartilage in osteoarthritis. *J Rheumatol* 32: 1156-1158; discussion 1158-1159, 2005.

20. **Calafat J, Janssen H, Stahle-Backdahl M, Zuurbier AE, Knol EF, and Egesten A.** Human monocytes and neutrophils store transforming growth factor-alpha in a subpopulation of cytoplasmic granules. *Blood* 90: 1255-1266, 1997.

21. **Castaneda S, Roman-Blas JA, Largo R, and Herrero-Beaumont G.** Subchondral bone as a key target for osteoarthritis treatment. *Biochem Pharmacol* 83: 315-323, 2011.

22. **Cole AG.** A review of diversity in the evolution and development of cartilage: the search for the origin of the chondrocyte. *Eur Cell Mater* 21: 122-129, 2011.

23. **D'Andrea P, Calabrese A, Capozzi I, Grandolfo M, Tonon R, and Vittur F.** Intercellular Ca2+ waves in mechanically stimulated articular chondrocytes. *Biorheology* 37: 75-83, 2000.

24. **Das SK and Farooqi A.** Osteoarthritis. *Best Pract Res Clin Rheumatol* 22: 657-675, 2008.

25. **Davis CG.** The many faces of epidermal growth factor repeats. *New Biol* 2: 410-419, 1990.

26. **de Larco JE and Todaro GJ.** Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci U S A* 75: 4001-4005, 1978.

27. **Del Fattore A, Teti A, and Rucci N.** Bone cells and the mechanisms of bone remodelling. *Front Biosci (Elite Ed)* 4: 2302-2321, 2012.

28. **Derynck R, Roberts AB, Winkler ME, Chen EY, and Goeddel DV.** Human transforming growth factor-alpha: precursor structure and expression in E. coli. *Cell* 38: 287-297, 1984.

29. **Farahat MN, Yanni G, Poston R, and Panayi GS.** Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. *Ann Rheum Dis* 52: 870-875, 1993.

30. **Felson DT.** Developments in the clinical understanding of osteoarthritis. *Arthritis Res Ther* 11: 203, 2009.

31. **Felson DT.** The course of osteoarthritis and factors that affect it. *Rheum Dis Clin North Am* 19: 607-615, 1993.

32. **Fiorentino L, Pertica C, Fiorini M, Talora C, Crescenzi M, Castellani L, Alema S, Benedetti P, and Segatto O.** Inhibition of ErbB-2 mitogenic and transforming activity by RALT, a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. *Mol Cell Biol* 20: 7735-7750, 2000.

33. **Goldring MB.** Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. *Best Pract Res Clin Rheumatol* 20: 1003-1025, 2006.

34. **Goldring MB and Goldring SR.** Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. *Ann N Y Acad Sci* 1192: 230-237, 2010.

35. **Goldring MB and Goldring SR.** Osteoarthritis. *J Cell Physiol* 213: 626-634, 2007.

36. **Guilak F.** The deformation behavior and viscoelastic properties of chondrocytes in articular cartilage. *Biorheology* 37: 27-44, 2000.

37. **Hackel PO, Gishizky M, and Ullrich A.** Mig-6 is a negative regulator of the epidermal growth factor receptor signal. *Biol Chem* 382: 1649-1662, 2001.

38. **Hackel PO, Zwick E, Prenzel N, and Ullrich A.** Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol* 11: 184-189, 1999.

39. **Hadjidakis DJ and Androulakis, II.** Bone remodeling. *Ann N Y Acad Sci* 1092: 385-396, 2006.

40. **Hall BK and Miyake T.** All for one and one for all: condensations and the initiation of skeletal development. *Bioessays* 22: 138-147, 2000.

41. **Hallbeck AL, Walz TM, Briheim K, and Wasteson A.** TGF-alpha and ErbB2 production in synovial joint tissue: increased expression in arthritic joints. *Scand J Rheumatol* 34: 204-211, 2005.

42. **Hannan MT, Felson DT, and Pincus T.** Analysis of the discordance between radiographic changes and knee pain in osteoarthritis of the knee. *J Rheumatol* 27: 1513-1517, 2000.

43. **Hashimoto M, Nakasa T, Hikata T, and Asahara H.** Molecular network of cartilage homeostasis and osteoarthritis. *Med Res Rev* 28: 464-481, 2008.

44. **Hinton R, Moody RL, Davis AW, and Thomas SF.** Osteoarthritis: diagnosis and therapeutic considerations. *Am Fam Physician* 65: 841-848, 2002.

45. **Hochberg MC.** Mortality in osteoarthritis. *Clin Exp Rheumatol* 26: S120-124, 2008.

46. **Huang K and Wu LD.** Aggrecanase and aggrecan degradation in osteoarthritis: a review. *J Int Med Res* 36: 1149-1160, 2008.

47. **Hunter DJ.** Osteoarthritis. *Best Pract Res Clin Rheumatol* 25: 801-814, 2011.

48. **Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH, and Merlino GT.** TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell* 61: 1137-1146, 1990.

49. **Jin N, Gilbert JL, Broaddus RR, Demayo FJ, and Jeong JW.** Generation of a Mig-6 conditional null allele. *Genesis* 45: 716-721, 2007.

50. **Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, and Fahmi H.** Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol* 7: 33-42, 2011.

51. **Karsenty G.** The complexities of skeletal biology. *Nature* 423: 316-318, 2003.

52. **Khatib AM, Lomri A, Mitrovic RD, and Moldovan F.** Articular chondrocyte aging and endothelin-1. *Cytokine* 37: 6-13, 2007.

53. **Khatib AM, Lomri A, Moldovan F, Fiet J, and Mitrovic DR.** Constitutive and inducible expression of endothelin-1 in primary rat articular chondrocyte culture. *Cytokine* 9: 556-562, 1997.

54. **Khatib AM, Siegfried G, Messai H, Moldovan F, and Mitrovic DR.** Mechanism of inhibition of endothelin-1-stimulated proteoglycan and collagen synthesis in rat articular chondrocytes. *Cytokine* 17: 254-261, 2002.

55. **Klein TJ, Malda J, Sah RL, and Hutmacher DW.** Tissue engineering of articular cartilage with biomimetic zones. *Tissue Eng Part B Rev* 15: 143-157, 2009.

56. **Klooster AR and Bernier SM.** Tumor necrosis factor alpha and epidermal growth factor act additively to inhibit matrix gene expression by chondrocyte. *Arthritis Res Ther* 7: R127-138, 2005.

57. **Kobayashi T and Kronenberg H.** Minireview: transcriptional regulation in development of bone. *Endocrinology* 146: 1012-1017, 2005.

58. **Krasnokutsky S, Samuels J, and Abramson SB.** Osteoarthritis in 2007. *Bull NYU Hosp Jt Dis* 65: 222-228, 2007.

59. **Kronenberg HM.** Developmental regulation of the growth plate. *Nature* 423: 332-336, 2003.

60. **Kronenberg HM.** PTHrP and skeletal development. *Ann N Y Acad Sci* 1068: 1-13, 2006.

61. **Kronenberg HM.** The role of the perichondrium in fetal bone development. *Ann N Y Acad Sci* 1116: 59-64, 2007.

62. **Kumar V, Bustin SA, and McKay IA.** Transforming growth factor alpha. *Cell Biol Int* 19: 373-388, 1995.

63. **Kusada J, Otsuka T, Matsui N, Hirano T, Asai K, and Kato T.** Immuno-reactive human epidermal growth factor (h-EGF) in rheumatoid synovial fluids. *Nihon Seikeigeka Gakkai Zasshi* 67: 859-865, 1993.

64. **Lajeunesse D.** The role of bone in the treatment of osteoarthritis. *Osteoarthritis Cartilage* 12 Suppl A: S34-38, 2004.

65. **Lajeunesse D and Reboul P.** Subchondral bone in osteoarthritis: a biologic link with articular cartilage leading to abnormal remodeling. *Curr Opin Rheumatol* 15: 628-633, 2003.

66. **Lalich M, McNeel DG, Wilding G, and Liu G.** Endothelin receptor antagonists in cancer therapy. *Cancer Invest* 25: 785-794, 2007.

67. **Lee DC, Fenton SE, Berkowitz EA, and Hissong MA.** Transforming growth factor alpha: expression, regulation, and biological activities. *Pharmacol Rev* 47: 51-85, 1995.

68. **Lee DC, Rose TM, Webb NR, and Todaro GJ.** Cloning and sequence analysis of a cDNA for rat transforming growth factor-alpha. *Nature* 313: 489-491, 1985.

69. **Loeser RF.** Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators, and aging collide. *Arthritis Rheum* 54: 1357-1360, 2006.

70. **Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, and Mirams M.** Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int J Biochem Cell Biol* 40: 46-62, 2008.

71. **Madtes DK, Raines EW, Sakariassen KS, Assoian RK, Sporn MB, Bell GI, and Ross R.** Induction of transforming growth factor-alpha in activated human alveolar macrophages. *Cell* 53: 285-293, 1988.

72. **Manacu CA, Martel-Pelletier J, Roy-Beaudry M, Pelletier JP, Fernandes JC, Shipkolye FS, Mitrovic DR, and Moldovan F.** Endothelin-1 in osteoarthritic chondrocytes triggers nitric oxide production and upregulates collagenase production. *Arthritis Res Ther* 7: R324-332, 2005.

73. **Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, and Dunn AR.** Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 73: 249-261, 1993.

74. **Marie PJ.** Transcription factors controlling osteoblastogenesis. *Arch Biochem Biophys* 473: 98-105, 2008.

75. **Matsuo K and Irie N.** Osteoclast-osteoblast communication. *Arch Biochem Biophys* 473: 201-209, 2008.

76. **Messai H, Khatib AM, Lebrun G, Aubin P, Florina M, Jean F, and Mitrovic DR.** Endothelin-1 in monolayer cultures of articular chondrocytes from young and old rats: regulation by growth factors and cytokines. *Mech Ageing Dev* 114: 37-48, 2000.

77. **Messai H, Panasyuk A, Khatib A, Barbara A, and Mitrovic DR.** Endothelin-1 receptors on cultured rat articular chondrocytes: regulation by age, growth factors, and cytokines, and effect on cAMP production. *Mech Ageing Dev* 122: 519-531, 2001.

78. **Mueller MB and Tuan RS.** Anabolic/Catabolic balance in pathogenesis of osteoarthritis: identifying molecular targets. *PM R* 3: S3-11, 2011.

79. **Ortega Mateo A and de Artinano AA.** Highlights on endothelins: a review. *Pharmacol Res* 36: 339-351, 1997.

80. **Poole AR.** An introduction to the pathophysiology of osteoarthritis. *Front Biosci* 4: D662-670, 1999.

81. **Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, and Laverty S.** Composition and structure of articular cartilage: a template for tissue repair. *Clin Orthop Relat Res*: S26-33, 2001.

82. **Roberts AB, Lamb LC, Newton DL, Sporn MB, De Larco JE, and Todaro GJ.** Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. *Proc Natl Acad Sci U S A* 77: 3494-3498, 1980.

83. **Robling AG, Castillo AB, and Turner CH.** Biomechanical and molecular regulation of bone remodeling. *Annu Rev Biomed Eng* 8: 455-498, 2006.

84. **Roy-Beaudry M, Martel-Pelletier J, Pelletier JP, M'Barek KN, Christgau S, Shipkolye F, and Moldovan F.** Endothelin 1 promotes osteoarthritic cartilage degradation via matrix metalloprotease 1 and matrix metalloprotease 13 induction. *Arthritis Rheum* 48: 2855-2864, 2003.

85. **Salaffi F, Carotti M, Stancati A, and Grassi W.** Health-related quality of life in older adults with symptomatic hip and knee osteoarthritis: a comparison with matched healthy controls. *Aging Clin Exp Res* 17: 255-263, 2005.

86. **Sandell LJ.** Etiology of osteoarthritis: genetics and synovial joint development. *Nat Rev Rheumatol* 8: 77-89, 2012.

87. **Sandell LJ and Aigner T.** Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 3: 107-113, 2001.

88. **Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL, and Lee DC.** Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell* 61: 1121-1135, 1990.

89. **Sasaki K, Hattori T, Fujisawa T, Takahashi K, Inoue H, and Takigawa M.** Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. *J Biochem* 123: 431-439, 1998.

90. **Scaltriti M and Baselga J.** The epidermal growth factor receptor pathway: a model for targeted therapy. *Clin Cancer Res* 12: 5268-5272, 2006.

91. **Scanzello CR and Goldring SR.** The role of synovitis in osteoarthritis pathogenesis. *Bone* 51: 249-257, 2012.

92. **Silva I and Branco JC.** Rank/Rankl/opg: literature review. *Acta Reumatol Port* 36: 209-218, 2011.

93. **Sinusas K.** Osteoarthritis: diagnosis and treatment. *Am Fam Physician* 85: 49-56, 2012.

94. **Stanton LA, Underhill TM, and Beier F.** MAP kinases in chondrocyte differentiation. *Dev Biol* 263: 165-175, 2003.

95. **Taskiran D, Stefanovic-Racic M, Georgescu H, and Evans C.** Nitric oxide mediates suppression of cartilage proteoglycan synthesis by interleukin-1. *Biochem Biophys Res Commun* 200: 142-148, 1994.

96. **Valdes AM and Spector TD.** Genetic epidemiology of hip and knee osteoarthritis. *Nat Rev Rheumatol* 7: 23-32, 2011.

97. **van der Waal JM, Terwee CB, van der Windt DA, Bouter LM, and Dekker J.** The impact of non-traumatic hip and knee disorders on health-related quality of life as

measured with the SF-36 or SF-12. A systematic review. *Qual Life Res* 14: 1141-1155, 2005.

98. **Wagner EF and Karsenty G.** Genetic control of skeletal development. *Curr Opin Genet Dev* 11: 527-532, 2001.

99. **Wong DT, Weller PF, Galli SJ, Elovic A, Rand TH, Gallagher GT, Chiang T, Chou MY, Matossian K, McBride J, and et al.** Human eosinophils express transforming growth factor alpha. *J Exp Med* 172: 673-681, 1990.

100. **Woolf AD and Pfleger B.** Burden of major musculoskeletal conditions. *Bull World Health Organ* 81: 646-656, 2003.

101. **Xu D, Makkinje A, and Kyriakis JM.** Gene 33 is an endogenous inhibitor of epidermal growth factor (EGF) receptor signaling and mediates dexamethasone-induced suppression of EGF function. *J Biol Chem* 280: 2924-2933, 2005.

102. **Zhang W, Moskowitz RW, Nuki G, Abramson S, Altman RD, Arden N, Bierma-Zeinstra S, Brandt KD, Croft P, Doherty M, Dougados M, Hochberg M, Hunter DJ, Kwoh K, Lohmander LS, and Tugwell P.** OARSI recommendations for the management of hip and knee osteoarthritis, Part II: OARSI evidence-based, expert consensus guidelines. *Osteoarthritis Cartilage* 16: 137-162, 2008.

103. **Zhang X, Pickin KA, Bose R, Jura N, Cole PA, and Kuriyan J.** Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. *Nature* 450: 741-744, 2007.

104. **Zhang Y and Jordan JM.** Epidemiology of osteoarthritis. *Clin Geriatr Med* 26: 355-369, 2010.

105. **Zhang YW, Su Y, Lanning N, Swiatek PJ, Bronson RT, Sigler R, Martin RW, and Vande Woude GF.** Targeted disruption of Mig-6 in the mouse genome leads to early onset degenerative joint disease. *Proc Natl Acad Sci U S A* 102: 11740-11745, 2005.

Chapter 2

2 Transforming growth factor alpha induces endothelin receptor A in osteoarthritis

This chapter has been reproduced from:

Usmani SE, Appleton CT, and Beier F. Transforming growth factor-alpha induces endothelin receptor A expression in osteoarthritis. *J Orthop Res* 30: 1391-1397, 2012.

2.1 Abstract

Previously, our lab identified transforming growth factor-alpha (TGFα) as a novel factor involved in osteoarthritis (OA) in a surgical model of the disease. In the same study, we also observed increased transcript levels for endothelin receptor A (ET(A)R), a known contributor to cartilage pathology. To investigate the connection between TGFα and endothelin signaling in OA, primary articular chondrocytes and osteochondral explants were isolated from Sprague Dawley rats and treated with vehicle or TGFα. Expression of ET(A)R protein and its encoding gene *Ednra* was assessed. Chondrocytes and cartilage explants were also treated with the endothelin receptor A/B antagonist Bosentan, in order to determine whether TGFα effects could be blocked. TGFα induced expression of ET(A)R protein and its encoding gene *Ednra*. In primary chondrocyte cultures, Bosentan did not block TGFα responses of the anabolic genes *Sox9*, *Agc1*, and *Col2a1*, but reduced the induction of *Mmp13* and *Ednra* transcripts by TGFα. In osteochondral explants, the inhibitor partially blocked TGFα reduction of type II collagen, as well as induction of MMP13 and type II collagen neoepitopes. TGFα induces ET(A)R expression in articular chondrocytes and receptor antagonism appears to block some TGFα -induced catabolic

effects in a three-dimensional organ culture system. Thus, TGFα may be a therapeutic target upstream of ET(A)R in OA.

2.2 Introduction

Osteoarthritis (OA) is the most prevalent disabling condition in western society and places a serious strain on our health care system (2). Clinical symptoms such as joint pain and decreased joint function can severely affect day to day life for patients, however no cure exists and current treatments only address symptoms without affecting the underlying causes (10). As a result, there is a significant need for further investigation and understanding of OA pathology.

While OA is a multivariable disease affecting the entire joint, one defining feature is the degeneration of articular cartilage (2). To better understand underlying pathological mechanisms, our lab has recently established a genome-wide profile of differentially expressed genes in degrading cartilage in a surgical rodent model of OA (5). Interestingly, transforming growth factor alpha (TGFα) transcript levels were upregulated in degenerating cartilage when compared to controls (5). Thus TGFα was identified as a novel candidate growth factor involved in cartilage degradation. Further exploration revealed that TGFα treatment had profound effects on the chondrocyte phenotype. For example, treatment of monolayer chondrocyte cell cultures with TGFα resulted in suppressed expression of anabolic genes including aggrecan and type II collagen while matrix metalloproteinase 13 (MMP13) gene expression increased (6). TGFα treatment also altered chondrocyte morphology and induced stress fibre formation in monolayer cultures (6). Articular cartilage organ culture studies revealed that cartilage treated with TGFα expressed more MMP13 than controls and also displayed other OA-like

characteristics such as cell clusters (6). A subset of human OA patients was also found to have higher than normal TGFα mRNA levels, thus establishing an important correlation between our rodent model and the human disease state (6).

TGFα is a member of the epidermal growth factor (EGF) family and contains the characteristic EGF-like domain which binds to the EGF receptor (16). While EGF itself was down-regulated in our experimental OA model, EGF receptor phosphorylation was increased, indicating receptor activation (6). Furthermore, a recent study showed that genetically modified mice with enhanced EGFR signaling spontaneously develop OA (32). EGFR signaling is also known to overlap and interact many other signaling systems, including the G-protein coupled receptor (GPCR) endothelin receptor A (ET(A)R) (11). In articular chondrocytes specifically, it has been observed that EGF induces expression of ET(A)R as well as of its ligand, endothelin-1 (ET-1) (19, 20). Our interest in interactions between these two signaling systems emerged firstly from the fact that, like TGFα, ET(A)R gene expression was upregulated in our animal model of OA (5) and secondly from the body of research implicating both systems in OA disease progression.

Previous studies have indicated that ET(A)R and its ligand play important roles in chondrocyte physiology and pathology. Studies using rodent articular chondrocytes showed that ET-1 production and ET(A)R density increased with age (14, 20). This trend is significant as age is the number one risk factor associated with OA (10). Prolonged exposure to ET-1 has also been shown to inhibit proteoglycan and collagen synthesis in rat articular chondrocytes (15). These data are complemented by reports which indicate that ET-1 increases expression of the matrix metalloproteinases MMP1 and MMP13 in human osteoarthritic chondrocytes (17, 23). Human osteoarthritic chondrocytes also

respond to ET-1 with increased expression of inducible nitric oxide synthase (iNOS), as well as elevated nitric oxide (NO) production (17). Elevated levels of iNOS are also characteristic in OA cartilage and studies have shown that inhibitors of iNOS can reduce the progression of experimental OA (3, 22, 27). Recently, Kaufman et al., elegantly demonstrated that antagonism of ET(A)R prevents articular cartilage degradation in a surgical rat model of OA (13). In this study, we hypothesized that TGFα signals upstream of ET(A)R and that inhibiting ET(A)R might block some of TGFα's OA-like changes in articular cartilage.

2.3 Materials and Methods

2.3.1 Surgical Model of Osteoarthritis

Tissues from our original surgical OA model were analyzed in this study. Weight controlled adult male Sprague Dawley rats underwent anterior cruciate ligament transection (ACL-T) and partial medial meniscectomy (PM) of the right knee joint or sham surgery as described (4, 12). All animals were exercised on a rotating cylinder for 30 minutes three times a week in order to allow full flexion and extension of their knee joints (4).

2.3.2 Histology and Immunohistochemistry

Organ culture explants were fixed overnight in 4% paraformaldehyde and then decalcified in an EDTA solution (0.4M EDTA, 0.3N NaOH, 1.5% glycerol and pH 7.3). Decalcification was determined by a physical end-point test. Tissues were processed, embedded in paraffin wax, and sectioned (6 μm thick) by the Molecular Pathology Laboratory at Robarts Research Institute (London, ON, Canada). Immunohistochemistry (IHC) was performed by first dewaxing sections in xylene and then rehydrating through a

series of graded ethanols ending in water. Antigen retrieval was performed in sodium citrate pH 6.0 for cellular proteins or Proteinase K for matrix-specific proteins. Tissues were then blocked in 5% serum and incubated with primary antibody overnight at 4^{o}C. Incubation with horseradish peroxidise-conjugated secondary antibody was followed by colorimetric detection with the substrate diaminodbenzidine (DAB). Primary antibodies used include anti-ET(A)R, -ET(B)R and -iNOS (Abcam, Cambridge, MA, USA), anti-type II collagen (R&D Systems, Minneapolis, MN, USA), anti-MMP13 (Cedarlane, Hornby, ON, Canada) and anti-type II collagen neoepitope (7, 9).

2.3.3 Cell Culture and Organ Culture Studies

All cell and organ culture reagents were purchased from Invitrogen (Burlington, Ont., Canada) and Sigma (Oakville, Ont., Canada) while sterile plates were purchased from BD Falcon (Mississauga, ON, Canada). All cells and explants were maintained in a 37^{o}C humidified incubator at 5% CO_2 and medium was changed daily.

2.3.4 Primary Articular Chondrocyte Cell Culture

Primary articular chondrocytes were isolated from the distal femoral condyles of neonatal Sprague Dawley rats as previously described and plated at a density of 5.0 x 10^4 cells/cm^2 (24). A 10% fetal bovine serum culture medium was prepared from a 2:3 ratio of DMEM:F12 supplemented with 50μg/ml ascorbic acid, 0.25% L-glutamine and 0.25% penicillin/streptomycin. Chondrocytes were serum starved for 24 hours prior to treatment and serum free medium was used throughout the remainder of the time course. Chondrocyte culture medium was supplemented with TGFα (final concentration 10 ng/ml) or an equal volume of vehicle (0.1% bovine serum albumin in PBS) for up to 72 hours. Culture medium was changed daily. For our endothelin inhibitor studies, cells

were treated with vehicle, TGFα (10 ng/ml), the endothelin receptor inhibitor Bosentan (10 μM; provided by Actelion Pharmaceuticals Ltd , Allschwil, Switzerland) or a combination of both for 48 hours. Bosentan is a dual endothelin receptor A/B antagonist which is clinically used for the treatment of pulmonary arterial hypertension (18).

2.3.5 E15.5 Tibia Organ Culture

Time-mated CD1 mice were purchased from Charles River Laboratory (St. Constant, Quebec, Canada) and embryonic E15.5 tibia were dissected as previously described (1, 26). Tibiae were cultured in media containing α-MEM supplemented with ascorbic acid, beta-glycerophosphate, bovine serum albumin, PENSTREP®, and L-glutamine. All tissues were maintained in a 37^{o}C humidified incubator at 5% CO_2 and treated with a range of concentrations of recombinant human TGFα (0 to 1000 ng/ml) for six days. Culture medium was changed every other day.

2.3.6 RNA Isolation and Real-Time PCR

RNA was isolated from rat primary chondrocytes using the Qiagen RNeasy Mini Kit and the manufacturer's animal cell protocol (Qiagen, Mississauga, ON, Canada). Mouse cartilage was dissected from the ends of tibia organ cultures with the aid of a Zeiss Stemi DV4 Stereo microscope as previously described (28), samples were placed in QIAzol solution (Qiagen, Mississauga, ON, Canada), and RNA was isolated following the manufacturer's protocol. Real-time PCR was performed for the genes *Ednra* (endothelin receptor A), *Il1b* (interleukin-1 beta), *Cxcr4* (chemokine (C-X-C motif) receptor 4), *Tgfa* (transforming growth factor alpha), *Agc1* (aggrecan), *Col2a1 (*type II collagen), *Sox9* (Sox9), and *Mmp13* (matrix metalloproteinase 13). Analysis was performed using the Applied Biosystems 7900HT Real-Time PCR system, the TaqMan® One-step Mastermix

Kit, and commercially available probes (Applied Biosystems, Foster City, CA, USA). All samples were normalized to the housekeeping gene *Gapdh* and day zero or vehicle-treated controls using the delta-delta cycle threshold (ΔΔCT) method.

2.3.7 Articular Cartilage Organ Culture

Osteochondral explants were isolated from the distal femoral condyles and proximal tibial plateaus of 4-5 month old adult male Sprague Dawley rats. Explants were then placed in 12-well tissue culture plates and submerged in a 2% bovine serum albumin culture medium made from alpha-minimal essential medium supplemented with 50μg/ml ascorbic acid, 0.25% L-glutamine and 0.25% penicillin/streptomycin for 24 hours prior to treatment. The same treatment groups described for primary chondrocyte experiments were repeated for organ culture studies. Explants were treated daily for up to 5 days before tissues were prepared for histology.

2.3.8 Rhodamine Phalloidin Staining

Primary chondrocyte cell cultures were isolated as described above and plated on micro cover glasses (VWR, Mississauga, ON, Canada) in 24-well plates at a density of 1.0 x 10^4 cells/cm^2. Cells were treated with one of four treatments: vehicle, TGFα (10 ng/ml), Bosentan (10 μM), or both for 48 hours. Cells were then fixed, permeabilized, and stained with rhodamine phalloidin (Cytoskeleton Inc., Denver, CO, USA) to visualize F-actin and overall cell morphology.

2.3.9 Statistics

All statistical analysis was performed using GraphPad Prism software Version 4.0. Real-time data was analysed using either a one-way analysis of variance (ANOVA) with a

Tukey's post-test or a two-way ANOVA with a Bonferroni's post-test. All graphs show mean values + standard error of the mean (SEM). A minimum of three independent groups was used in all experiments.

2.4 Results

2.4.1 ET(A)R expression increases in a surgical model of osteoarthritis

Our previous studies have shown that ET(A)R gene expression is upregulated in OA-operated animals compared to controls, as demonstrated by microarrays and validated by real-time PCR (5). We next wanted to examine ET(A)R protein expression in histological samples from OA- and sham-operated animals. Immunohistochemical analysis revealed more ET(A)R signal throughout articular surfaces in OA animals compared to controls (Figure 2.1).

2.4.2 TGFα treatment induces ET(A)R gene expression

To examine whether TGFα can induce *Ednra* expression, primary rat chondrocytes in monolayer culture were treated with vehicle or TGFα (10 ng/ml) for 1 – 3 days, followed by isolation of RNA and real-time PCR analyses. *Ednra* mRNA expression increased after 2 and 3 days of TGFα treatment (Figure 2.2A). Day 2 showed about a 9-fold increase while day 3 showed about a 6-fold increase (Figure 2.2A). In contrast, transcript levels of several other genes known to be involved in OA or identified in our microarray, including *Tgfa* itself*, Il1b*, and *Cxcr4,* did not change significantly after treatment (Figure 2.2B-D). We next wanted to determine whether TGFα-induction of *Ednra* was species specific. We therefore employed the mouse E15.5 tibia organ culture system. RNA isolated from the cartilage ends of TGFα-treated tibiae also showed an induction of

Figure 2.1 Endothelin receptor A (ET(A)R) protein expression is upregulated in degenerating cartilage from an experimental rodent model of osteoarthritis (OA). Histological sections were prepared from knee joint articular cartilage of both sham and OA operated rats 4 weeks post-surgery. Immunohistochemistry was performed with an anti-ET(A)R primary antibody followed by secondary antibody and visualization with the substrate DAB (brown precipitate). Nuclei were counterstained with hematoxylin (blue). Representative images show more ET(A)R staining in OA cartilage compared to sham cartilage. This experiment was performed on three separate trials with similar results and one representative trial is shown.

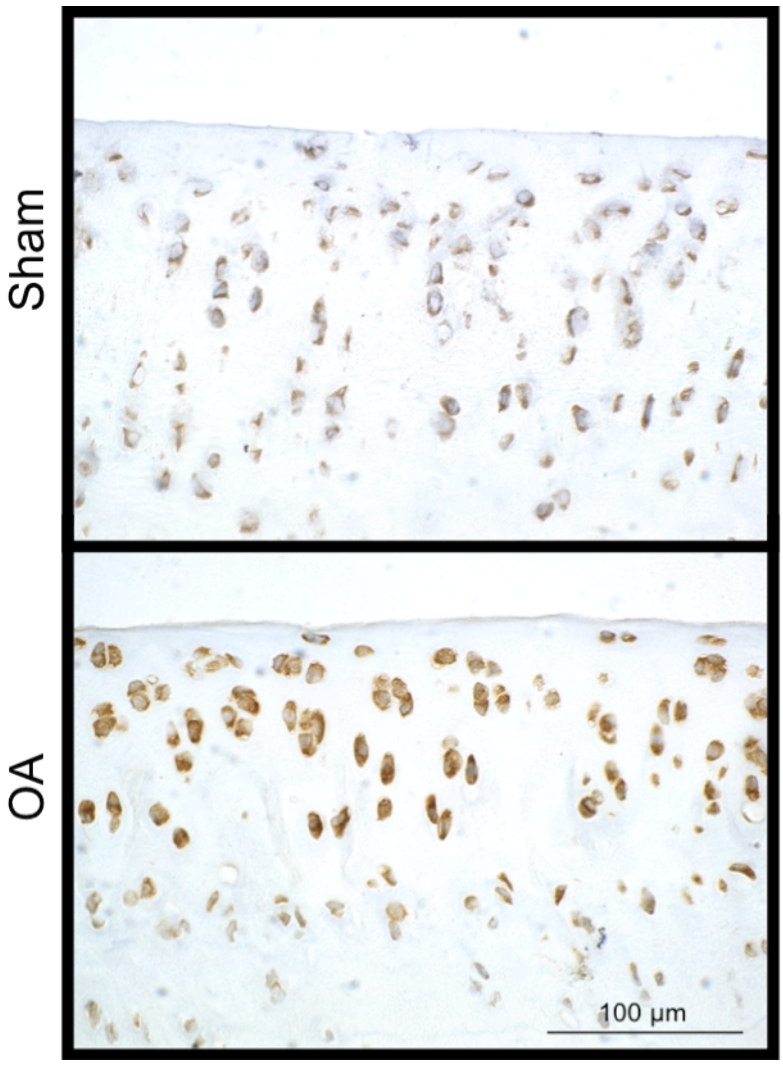


Figure 2.1 Endothelin receptor A (ET(A)R) protein expression is upregulated in degenerating cartilage from an experimental rodent model of osteoarthritis (OA).

Figure 2.2 TGFα treatment increases expression of the endothelin receptor A gene (*Ednra*). Neonatal rat primary chondrocytes were treated with or without TGFα (10 ng/ml) for up to 3 days (A-D) while E15.5 CD1 mouse tibiae were treated with a range of TGFα concentrations (0-1000 ng/ml) for 6 days (E). Real-time PCR was used to determine gene expression relative to *Gapdh* and day 0 controls. Relative expression means +SEM are shown. A) *Ednra* mRNA expression increased in rat primary cultures after days 2 and 3 of treatment while expression of the genes *Il1b*, *Cxcr4* and *Tgfa* did not change significantly after treatment (B-D). Treated mouse tibiae also showed increased levels of *Ednra* transcripts (n=3-4, *P<0.05).

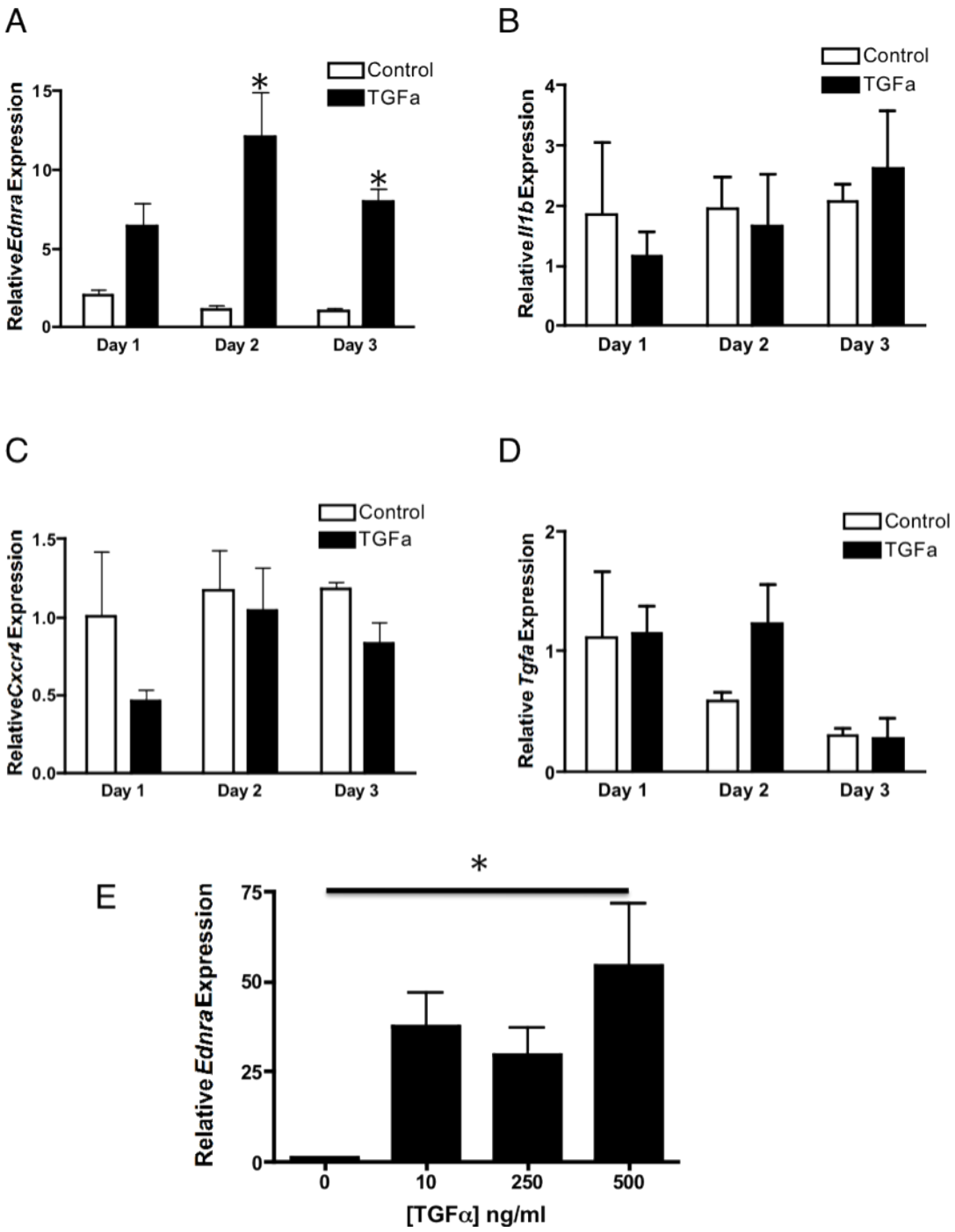


Figure 2.2 TGFα treatment increases expression of the endothelin receptor A gene (*Ednra*).

Ednra transcripts, although a higher dose of TGFα was required to reach significance (Figure 2.2E).

2.4.3 TGFα treatment induces ET(A)R protein expression

To study TGFα's effects on ET(A)R expression in a more physiological three-dimensional context, articular organ culture explants were isolated from male Sprague Dawley rats and treated with or without TGFα. Tissues were immunostained for ET(A)R and ET(B)R and representative images from day 1 and day 5 are shown (Figure 2.3A). Figure 2.3 reveals an increase in ET(A)R staining in TGFα-treated explants compared to controls. No evident change in ET(B)R expression was observed between treatments and controls (Figure 2.3A).

2.4.4 iNOS, and NF-κB expression increase with TGFα treatment

Endothelin signalling is known to activate iNOS expression (17). Tissue sections from articular cartilage organ cultures treated with vehicle or TGFa were stained with antibodies against iNOS and the p65 subunit of NF-κB (a transcription factor regulating iNOS expression (21)). The TGFα-treated tissues appear to have more positively stained cells for iNOS than controls after 5 days of treatment (Figure 2.3B). In particular, staining extended deeper into the cartilage in TGFα-treated samples. We saw a similar trend for NF-κB staining (Figure 2.3B).

2.4.5 Bosentan treatment does not block TGFα-induced gene changes

To address whether inhibition of endothelin receptors can block effects of TGFα on gene expression, primary chondrocytes were treated with vehicle, TGFα, the endothelin receptor A/B inhibitor Bosentan or both. Bosentan treatment did not block TGFα-

Figure 2.3 Endothelin receptor A (ET(A)R) expression increases in TGFα-treated articular cartilage explants. Tibial and femoral articular cartilage explants were isolated from Sprague Dawley rats and cultured with or without TGFα (10 ng/ml) for up to 5 days. Histological sections were then immunostained for ET(A)R, ET(B)R, iNOS and NF-kB (brown precipitate) and counterstained with hematoxylin (blue). A) Representative images show that ET(A)R expression increased after 5 days of treatment while ET(B)R showed no change with TGFα treatment. B) TGFα-treated tissues show more staining for both iNOS and the transcription factor NF-kB. This experiment was performed on three separate trials with similar results and one representative trial is shown.

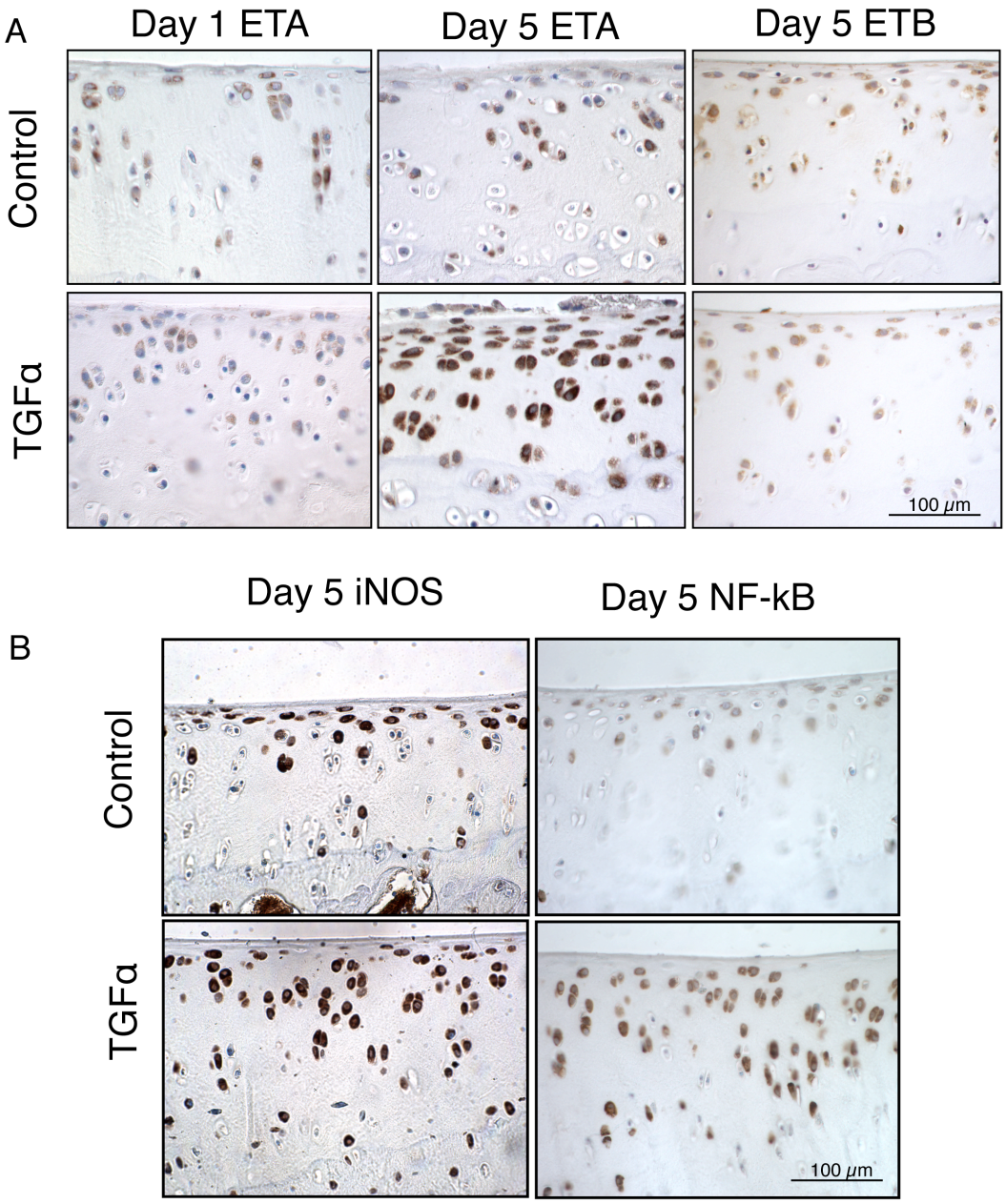


Figure 2.3 Endothelin receptor A (ET(A)R) expression increases in TGFα-treated articular cartilage explants.

suppression of *Agc1*, *Col2a1, or Sox9 mRNA levels* (Figure 2.4A-C); however, Bosentan slightly decreased the induction of *Ednra* (Figure 2.4D) and *Mmp13* (Figure 2.4E). In the absence of TGFα, Bosentan did not induce any significant changes in chondrocyte gene expression.

2.4.6 Bosentan treatment does not block TGFα effects on cell morphology

TGFα has been shown to alter cell shape and induce stress fiber formation in monolayer articular chondrocyte cultures (6), in agreement with loss of a chondrocyte phenotype (31). As in our earlier studies, TGFα induced stress fiber formation and a more elongated cell shape in primary chondrocytes (Figure 2.5). By itself, Bosentan did not appear to have any effects on cell morphology or actin organization (Figure 2.5). Moreover, when Bosentan treatment was given in combination with TGFα, no major change was observed when compared to TGFα treatment alone (Figure 2.5).

2.4.7 Bosentan treatment blocks TGFα effects on cartilage ECM turnover

After finding limited effects of endothein receptor A/B inhibition in our monolayer articular chondrocyte model, we decided to perform inhibitor studies in the more physiologically relevant organ culture model. Explants were cultured with vehicle, TGFα, Bosentan or both. Tissue sections were then immunostained for type II collagen, MMP13, and type II collagen neoepitopes that become detectable when type II collagen is cleaved by MMP13. TGFα-treated tissues showed a decrease in overall type II collagen staining in the extracellular matrix (ECM) (Figure 2.6A) and an increase in MMP13 staining (Figure 2.6B). Co-treatment with Bosentan appeared to reverse these catabolic effects (Figure 2.6A,B). Furthermore, TGFα-treatment appeared to generate more type II

Figure 2.4 Bosentan treatment does not block TGFα-effects on gene expression. Primary articular chondrocytes were cultured with vehicle, TGFα (10 ng/ml), the endothelin receptor A/B inhibitor Bosentan (10 μM), or a combination of both for 48 hours. RNA was isolated and real-time PCR was performed for the genes *Acg1*, *Col2a1*, *Sox9*, *Ednra*, and *Mmp13*. For all genes, measurements were relative to *Gapdh* and vehicle-treated controls. Bosentan and had a slight effect of induction of *Ednra* (D) and *Mmp13* (E). Bosentan did not have a significant effect on any other genes (A, B, C). Relative expression means +SEM are shown. Means with different letters are significantly different (n=4, $P<0.05$).

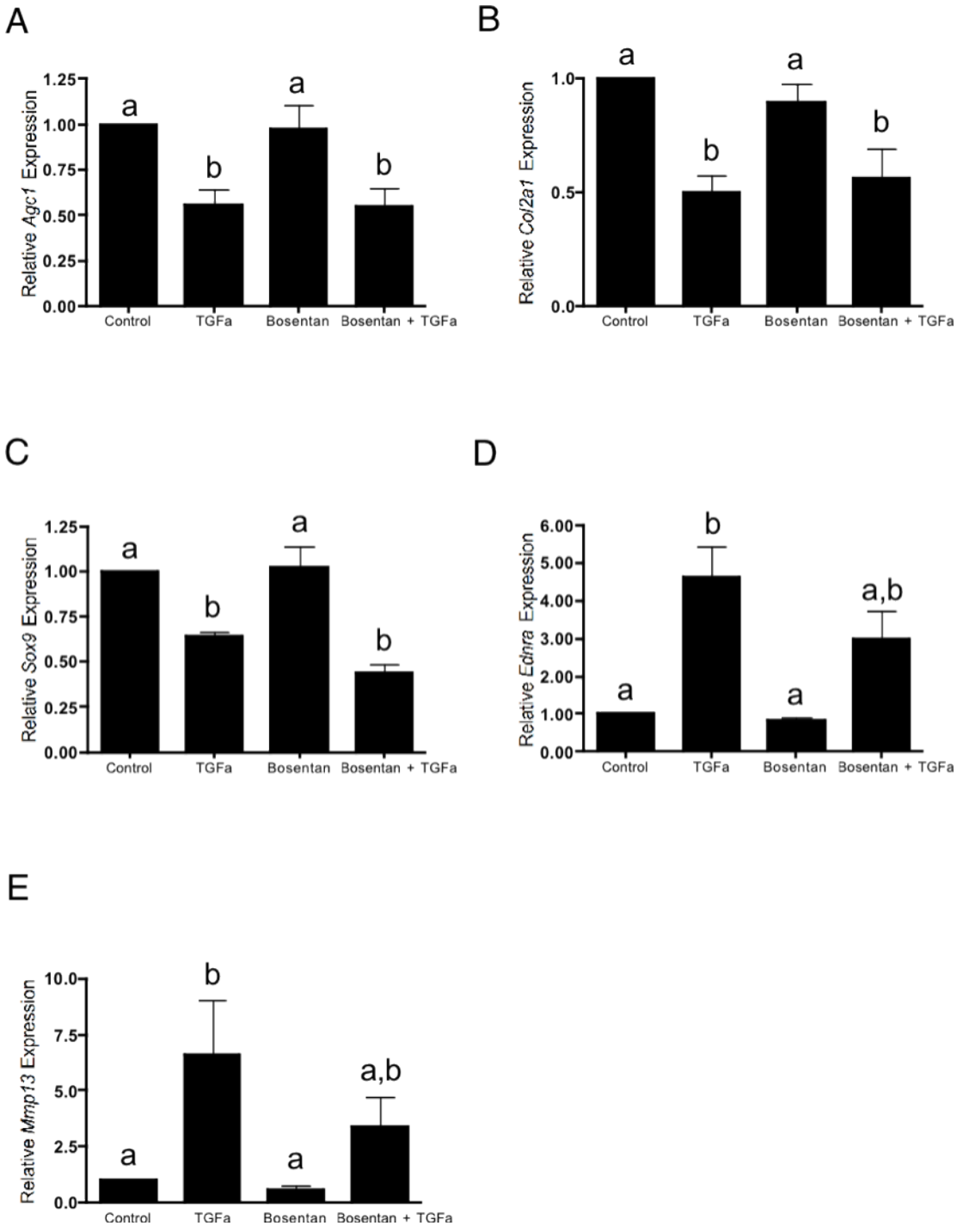


Figure 2.4 Bosentan treatment does not block TGFα-effects on gene expression.

Figure 2.5 Bosentan treatment does not alter TGFα effects on actin organization. Primary articular chondrocytes were cultured and treated with vehicle, TGFα (10 ng/ml), the endothelin receptor inhibitor A/B Bosentan (10 μM), or a combination of both for 48 hours. Cells were then fixed and stained with rhodamine phalloidin (red) in order to visualize F-actin. Nuclei were stained with DAPI (blue). Representative images of each treatment are shown (n=3). Bosentan does not block TGFα effects on cell morphology or induction of stress fibers.

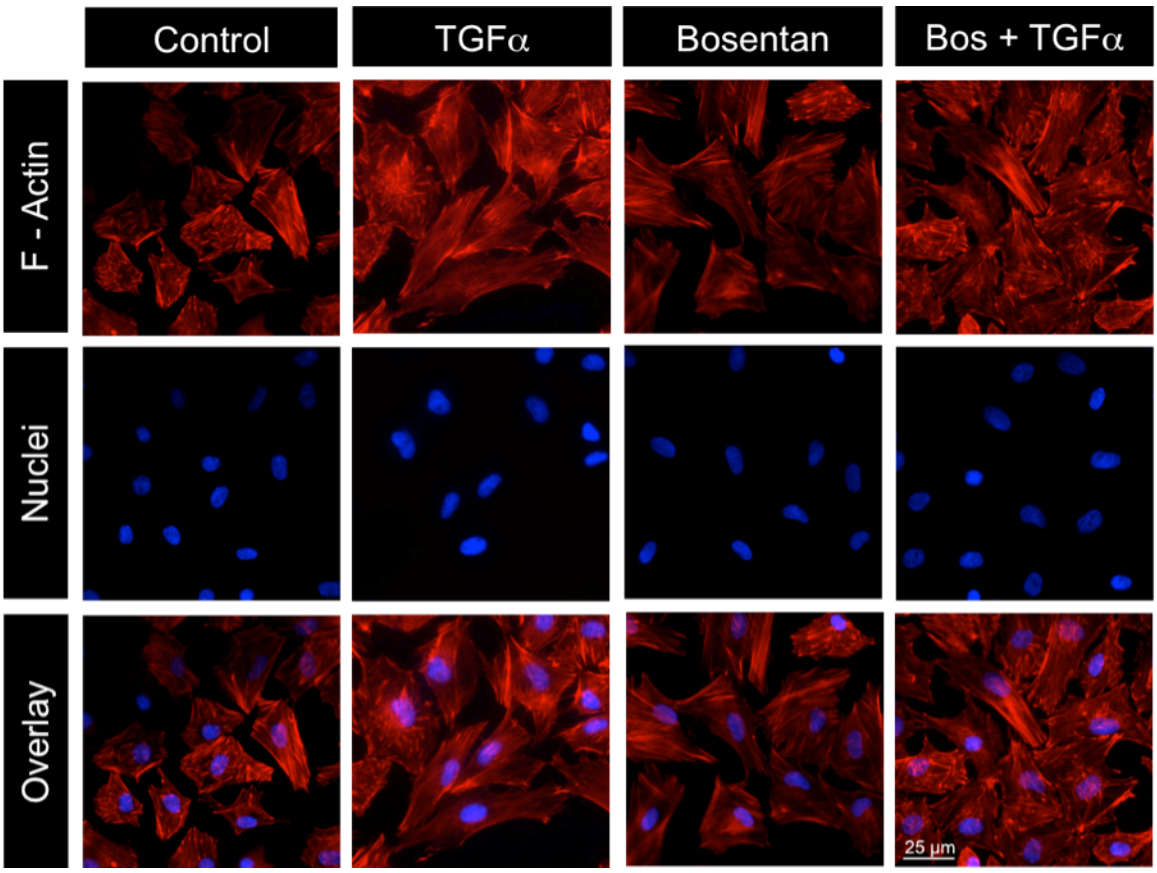


Figure 2.5 Bosentan treatment does not alter TGFα effects on actin organization.

Figure 2.6 Bosentan treatment blocks TGFα reduction of type II collagen, induction of MMP13, and induction of type II collagen neoepitopes. Articular cartilage explants were treated with vehicle, TGFα (10 ng/ml), the endothelin receptor A antagonist Bosentan (10 μM), or both for 5 days. Tissues were then immunostained for type II collagen (A), MMP13 (B), and type II collagen neoepitopes (C) (brown precipitate). TGFα-treated tissues showed a decrease in type II collagen staining, an increase in MMP13 staining, and an increase in type II collagen neoepitopes. Co-treatment with TGFα and Bosentan appeared to bring expression levels back to control levels. This experiment was performed on four separate trials with similar results and one representative trial is shown.

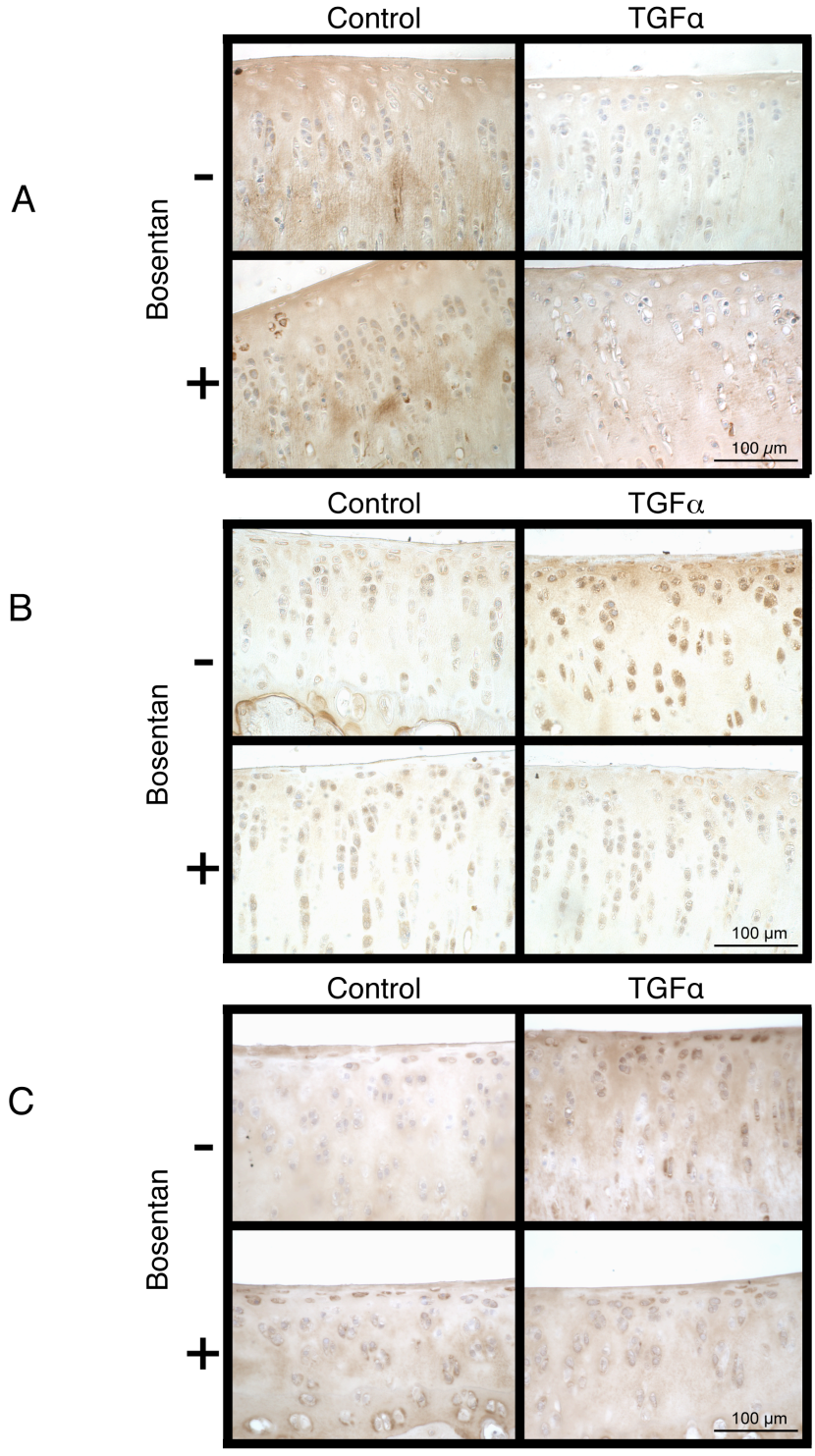


Figure 2.6 Bosentan treatment blocks TGFα reduction of type II collagen, induction of MMP13, and induction of type II collagen neoepitopes.

collagen neoepitopes in the ECM when compared to controls, but the combination with Bosentan appeared to block neoepitope induction (Figure 2.6C).

2.5 Discussion

We have previously identified TGFα as a growth factor capable of inducing OA-like phenotypic changes in articular chondrocytes. In this study, we demonstrate that TGFα induces ET(A)R expression in articular chondrocytes at both the gene and protein level. Furthermore, we demonstrate that TGFα-induction of the receptor occurs in both rat and mouse models as well as in varying ages of tissue (embryonic, newborn, and four-five month-old). This is consistent with previous findings, in which EGF was able to induce ET(A)R expression in both young and old rats (20). In addition to EGF and TGFα, a number of other growth factors and cytokines are known to modulate ET(A)R receptor density in chondrocytes (20). Furthermore, the literature suggests that the ET(A)R receptor plays a role in both aging and degenerative joint disease (14, 17, 20, 23). Thus, TGFα induction of ET(A)R might be one way through which TGFα mediates its deleterious effects.

To test this hypothesis, we used the dual endothelin receptor A/B inhibitor Bosentan in combination with TGFα treatment. We found that Bosentan treatment was not sufficient to block TGFα-effects on anabolic gene expression in monolayer culture. Bosentan did, however, have a minor effect on *Mmp13* and *Ednra* levels. Bosentan was also unable to reverse TGFα-induced changes to the chondrocyte actin cytoskeleton and cell shape. This leads us to believe that other downstream pathways might be responsible for anabolic gene expression changes and cytoskeletal rearrangement in TGFα-treated

chondrocytes. Previous studies done in our laboratory show that TGFα activates many intracellular signalling pathways (7). For example, RhoA/ROCK mediates TGFα-induced morphologic changes in chondrocytes, in agreement with our previous studies demonstrating important roles of this pathway in chondrogenesis (29, 30). The MEK/ERK pathway mediates the down regulation of anabolic gene expression by TGFα (7), again line with the known roles of this pathway in chondrocytes (reviewed in (8, 25)). Both Rho/ROCK and MEK/ERK pathways also regulate type II collagen cleavage and aggrecan breakdown in articular cartilage (7).

Bosentan had a stronger effect in the three-dimensional organ culture system where endothelin receptor A/B inhibition was able to suppress TGFα induction of the catabolic factor MMP13 and subsequent type II collagen break down. There are some possible explanations for the differences observed in our two experimental systems. Firstly, age may play a role: primary cells used in our studies were isolated from neonatal rats while organ culture explants were isolated from adult rats. The baseline density of ET(A)R receptors is known to be dependent on the age of articular chondrocytes and more ET-1 is known to be produced in older chondrocytes (14, 20). In addition, endothelin receptor signaling might be more important in the authentic three-dimensional context of cartilage than in monolayer culture. Recently, Kaufman et al. showed that ET(A)R inhibition prevented OA progression in a surgical model of the disease (13), in agreement with our data shown here.

In our studies, we observed no effect on any parameters with Bosentan treatment alone. While articular chondrocytes produce ET-1, it is possible that its levels are not high enough in the *in vitro* environment to sufficiently stimulate ET(A)R receptors. ET-1

mRNA levels however, did not change in the TGFα treatment groups (data not shown), suggesting that it is the receptor density itself more so than the concentration of ligand that is responsible for enhanced signaling.

In summary, our data show that ET(A)R expression in articular cartilage is increased in response to both surgical induction of OA and TGFα treatment. We also demonstrate that this pathway is only partially responsible for TGFα-induction of an OA-like phenotype, but that ET(A)R inhibition suppresses catabolic activities in articular cartilage. Further studies should be done to examine the potential benefit of upstream targets such at the EGF receptor itself as well as combinations of downstream targets in OA therapy.

2.6 References

1. **Agoston H, Khan S, James CG, Gillespie JR, Serra R, Stanton LA, and Beier F.** C-type natriuretic peptide regulates endochondral bone growth through p38 MAP kinase-dependent and -independent pathways. *BMC Dev Biol* 7: 18, 2007.

2. **Aigner T, Sachse A, Gebhard PM, and Roach HI.** Osteoarthritis: pathobiology-targets and ways for therapeutic intervention. *Adv Drug Deliv Rev* 58: 128-149, 2006.

3. **Amin AR, Di Cesare PE, Vyas P, Attur M, Tzeng E, Billiar TR, Stuchin SA, and Abramson SB.** The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: evidence for up-regulated neuronal nitric oxide synthase. *J Exp Med* 182: 2097-2102, 1995.

4. **Appleton CT, McErlain DD, Pitelka V, Schwartz N, Bernier SM, Henry JL, Holdsworth DW, and Beier F.** Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. *Arthritis Res Ther* 9: R13, 2007.

5. **Appleton CT, Pitelka V, Henry J, and Beier F.** Global analyses of gene expression in early experimental osteoarthritis. *Arthritis Rheum* 56: 1854-1868, 2007.

6. **Appleton CT, Usmani SE, Bernier SM, Aigner T, and Beier F.** Transforming growth factor alpha suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. *Arthritis Rheum* 56: 3693-3705, 2007.

7. **Appleton CT, Usmani SE, Mort JS, and Beier F.** Rho/ROCK and MEK/ERK activation by transforming growth factor-alpha induces articular cartilage degradation. *Lab Invest* 90: 20-30, 2010.

8. **Beier F and Loeser RF.** Biology and pathology of Rho GTPase, PI-3 kinase-Akt, and MAP kinase signaling pathways in chondrocytes. *J Cell Biochem* 110: 573-580, 2010.

9. **Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, and Poole AR.** Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99: 1534-1545, 1997.

10. **Buckwalter JA and Martin JA.** Osteoarthritis. *Adv Drug Deliv Rev* 58: 150-167, 2006.

11. **Hackel PO, Zwick E, Prenzel N, and Ullrich A.** Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol* 11: 184-189, 1999.

12. **Hayami T, Pickarski M, Zhuo Y, Wesolowski GA, Rodan GA, and Duong le T.** Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. *Bone* 38: 234-243, 2006.

13. **Kaufman GN, Zaouter C, Valteau B, Sirois P, and Moldovan F.** Nociceptive tolerance is improved by bradykinin receptor B1 antagonism and joint morphology is protected by both endothelin type A and bradykinin receptor B1 antagonism in a surgical model of osteoarthritis. *Arthritis Res Ther* 13: R76, 2011.

14. **Khatib AM, Lomri A, Mitrovic RD, and Moldovan F.** Articular chondrocyte aging and endothelin-1. *Cytokine* 37: 6-13, 2007.

15. **Khatib AM, Siegfried G, Messai H, Moldovan F, and Mitrovic DR.** Mechanism of inhibition of endothelin-1-stimulated proteoglycan and collagen synthesis in rat articular chondrocytes. *Cytokine* 17: 254-261, 2002.

16. **Kumar V, Bustin SA, and McKay IA.** Transforming growth factor alpha. *Cell Biol Int* 19: 373-388, 1995.

17. **Manacu CA, Martel-Pelletier J, Roy-Beaudry M, Pelletier JP, Fernandes JC, Shipkolye FS, Mitrovic DR, and Moldovan F.** Endothelin-1 in osteoarthritic chondrocytes triggers nitric oxide production and upregulates collagenase production. *Arthritis Res Ther* 7: R324-332, 2005.

18. **Mathier MA and Ishizawar D.** Bosentan. *Expert Opin Pharmacother* 11: 1023-1034, 2010.

19. **Messai H, Khatib AM, Lebrun G, Aubin P, Florina M, Jean F, and Mitrovic DR.** Endothelin-1 in monolayer cultures of articular chondrocytes from young and old rats: regulation by growth factors and cytokines. *Mech Ageing Dev* 114: 37-48, 2000.

20. **Messai H, Panasyuk A, Khatib A, Barbara A, and Mitrovic DR.** Endothelin-1 receptors on cultured rat articular chondrocytes: regulation by age, growth factors, and cytokines, and effect on cAMP production. *Mech Ageing Dev* 122: 519-531, 2001.

21. **Pautz A, Art J, Hahn S, Nowag S, Voss C, and Kleinert H.** Regulation of the expression of inducible nitric oxide synthase. *Nitric Oxide* 23: 75-93, 2010.

22. **Pelletier JP, Jovanovic DV, Lascau-Coman V, Fernandes JC, Manning PT, Connor JR, Currie MG, and Martel-Pelletier J.** Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. *Arthritis Rheum* 43: 1290-1299, 2000.

23. **Roy-Beaudry M, Martel-Pelletier J, Pelletier JP, M'Barek KN, Christgau S, Shipkolye F, and Moldovan F.** Endothelin 1 promotes osteoarthritic cartilage degradation via matrix metalloprotease 1 and matrix metalloprotease 13 induction. *Arthritis Rheum* 48: 2855-2864, 2003.

24. **Seguin CA and Bernier SM.** TNFalpha suppresses link protein and type II collagen expression in chondrocytes: Role of MEK1/2 and NF-kappaB signaling pathways. *J Cell Physiol* 197: 356-369, 2003.

25. **Stanton LA, Underhill TM, and Beier F.** MAP kinases in chondrocyte differentiation. *Dev Biol* 263: 165-175, 2003.

26. **Ulici V, Hoenselaar KD, Gillespie JR, and Beier F.** The PI3K pathway regulates endochondral bone growth through control of hypertrophic chondrocyte differentiation. *BMC Dev Biol* 8: 40, 2008.

27. **van der Harst M, Bull S, Brama PA, Barneveld AB, van Weeren PR, and van de Lest C.** Nitrite and nitrotyrosine concentrations in articular cartilage, subchondral bone, and trabecular bone of normal juvenile, normal adult, and osteoarthritic adult equine metacarpophalangeal joints. *J Rheumatol* 33: 1662-1667, 2006.

28. **Wang G, Woods A, Agoston H, Ulici V, Glogauer M, and Beier F.** Genetic ablation of Rac1 in cartilage results in chondrodysplasia. *Dev Biol* 306: 612-623, 2007.

29. **Wang G, Woods A, Sabari S, Pagnotta L, Stanton LA, and Beier F.** RhoA/ROCK signaling suppresses hypertrophic chondrocyte differentiation. *J Biol Chem* 279: 13205-13214, 2004.

30. **Woods A and Beier F.** RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. *J Biol Chem* 281: 13134-13140, 2006.

31. **Woods A, Wang G, and Beier F.** Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. *J Cell Physiol* 213: 1-8, 2007.

32. **RW, and Vande Woude GF.** Targeted disruption of Mig-6 in the mouse genome leads to early onset degenerative joint disease. *Proc Natl Acad Sci U S A* 102: 11740-11745, 2005.

Chapter 3

3 Transforming growth factor alpha controls the transition from hypertrophic cartilage to bone during endochondral bone growth

This chapter has been reproduced from:

Usmani SE, Pest MA, Kim G, Ohora SN, Qin L, and Beier F. Transforming growth factor alpha controls the transition from hypertrophic cartilage to bone during endochondral bone growth. *Bone* 51: 131-141, 2012.

3.1 Abstract

We have recently identified transforming growth factor alpha (TGFα) as a novel growth factor involved in the joint disease osteoarthritis. The role of TGFα in normal cartilage and bone physiology however, has not been well defined. The objective of this study was to determine the role of TGFα in bone development through investigation of the *Tgfa* knockout mouse. The gross skeletons as well as the cartilage growth plates of *Tgfa* knockout mice and their control littermates were examined during several developmental stages ranging from newborn to ten-weeks-old. Knockout mice experienced skeletal growth retardation and expansion of the hypertrophic zone of the growth plate. These phenotypes were transient and spontaneously resolved by ten weeks of age. *Tgfa* knockout growth plates also had fewer osteoclasts along the cartilage/bone interface. Furthermore, knockout mice expressed less RUNX2, RANKL, and MMP13 mRNA in their cartilage growth plates than controls. In conclusion, *Tgfa* knockout mice experience a delay in bone development, specifically the conversion of hypertrophic cartilage to true bone. The persistence of the hypertrophic zone of the growth plate appears to be mediated by a decrease in MMP13 and RANKL expression in hypertrophic chondrocytes and a resulting reduction in osteoclast recruitment. Overall, TGFα appears to be an

important growth factor regulating the conversion of cartilage to bone during the process of endochondral ossification.

3.2 Introduction

Our lab has recently identified transforming growth factor alpha (TGFα) as a novel factor involved in the degeneration of articular cartilage during osteoarthritis (3-5). Specifically, TGFα treatment modifies articular chondrocyte gene expression by decreasing levels of anabolic genes such as type II collagen (*Col2a1*) and aggrecan (*Agc1*) and increasing levels of catabolic genes such as matrix metalloproteinase 13 (*Mmp13*) (4). While much of our efforts have focused on TGFα's role in joint pathology, its role in normal bone and cartilage physiology, in particular during endochondral ossification, needs to be understood to fully appreciate its function in disease.

Endochondral ossification is a highly regulated process that begins when mesenchymal stem cells condense and differentiate into chondrocytes, providing an initial cartilage template that will eventually be replaced by bone (26, 28). Within the condensations, chondrocytes proliferate and secrete a characteristic matrix composed primarily of type II collagen and aggrecan (6, 28). Cells at the centre of the condensations stop proliferating, undergo hypertrophy, and secrete a matrix rich in type X collagen (28, 31). The hypertrophic chondrocytes attract blood vessels, direct the mineralization of their surrounding matrix, and ultimately undergo apoptosis (31). Osteoclasts and osteoblasts are then recruited in order to resorb hypertrophic cartilage and to replace it with true bone, respectively (26, 31). Eventually, secondary ossification centres form near the ends of developing bones, and the area between the two ossification centres is known as the cartilage growth plate (28). The growth plate consists of distinct layers, namely the

resting zone, the proliferating zone, and the hypertrophic zone (31). Proliferation of chondrocytes and subsequent cell enlargement during chondrocyte hypertrophy are the main drivers of endochondral bone growth (7) .

Several studies have suggested a potential role of TGFα in early bone development. TGFα is a member of the epidermal growth factor (EGF) family and contains the characteristic EGF-like domain which allows it to bind to and signal through the EGF receptor (EGFR) (29). Both TGFα and EGF negatively regulate chondrogenesis of limb bud mesenchymal cells during embryonic development (10, 16, 51). Other studies have examined long bone development in embryonic and neonatal EGFR null mice and found delayed endochondral ossification, inhibited osteoclast recruitment, and an expanded hypertrophic zone of the growth plate (49). Since EGFR null mice die soon after birth, postnatal development could not be assessed. Another group studied the effects of reduced EGFR signaling in older animals by creating a conditional knockin mouse humanized for *Egfr* (39). These mice survived up to six months of age, experienced growth retardation, as well as accelerated chondrocyte and osteoblast differentiation (39). Since both of these models target the EGF receptor itself, the identity of the physiological ligand(s) responsible for the aforementioned effects remains unknown.

TGFα has also been implicated in bone maintenance and turnover. Human recombinant TGFα stimulates bone resorption and inhibits bone formation *in vitro* (17, 18). In these studies fetal rat long bones and neonatal mouse calvariae were cultured and resorption was assessed by calcium release while bone formation was assessed by collagen synthesis and alkaline phosphatase activity (17, 18). These results are similar to those for EGF treatment, but TGFα appears to be a more potent agent, inducing the same results with a

more rapid time course (17). Tumor-derived TGFα also causes calcium release *in vitro,* supporting the hypothesis that TGFα may be responsible for the increased bone resorption and hypercalcemia seen in some malignancies (18). Additional studies have attempted to identify the mechanisms through which TGFα mediates its effects in bone. TGFα treatment of human marrow cultures was found to stimulate the formation of multinucleated osteoclast-like cells by encouraging the proliferation of precursor cells (42). Furthermore, co-cultures of osteoblastic cells and bone marrow macrophages (pre-osteoclasts) showed that TGFα treatment stimulates osteoclastogenesis by regulating osteoblast production of osteoprotegerin (OPG) and monocyte chemoattractant protein 1 (MCP1, also called CCL2) (53).

TGFα and its receptor appear to be important mediators of bone development and physiology. However, the role of TGFα in endochondral ossification and growth plate physiology has not been clearly defined. *Tgfa* knockout mice were initially characterized in 1993 and the main phenotypes reported include wavy fur, disorganized hair follicle arrangement, curly whiskers, and corneal inflammation (32). There have been no reports of cartilage and/or bone abnormalities. In this study, we examined the role of TGFα in endochondral ossification and characterized the growth plate phenotype of *Tgfa* null mice.

3.3 Materials and Methods

3.3.1 Materials

All cell and organ culture media reagents were purchased from Invitrogen (Burlington, ON, Canada) and Sigma (Oakville, ON, Canada) while sterile plates were purchased from BD Falcon (Mississauga, ON, Canada).

3.3.2 E15.5 tibia organ culture

Time-mated CD1 mice were purchased from Charles River Laboratory (St. Constant, Quebec, Canada). Tibiae were dissected from embryonic 15.5-day-old (E15.5) CD1 mice and cultured in media containing α-MEM supplemented with ascorbic acid, beta-glycerophosphate, bovine serum albumin, PENSTREP®, and L-glutamine as described previously (1, 44). Tibiae were maintained in a 37^{o}C humidified incubator at 5% CO_2 and cultured for six days. Tissues were treated with a range of concentrations of recombinant human TGFα (0 to 1000 ng/ml) every other day. On day one and day six tibiae were measured using a Zeiss Stemi DV4 Stereo microscope equipped with an eyepiece ruler. Growth was determined by subtracting the length on day 1 from the length on day 6. Four independent trials were averaged, and mean growth was analyzed using a one-way ANOVA with Tukey's post-tests. Whole tibiae were stained with alcian blue and alizarin red as previously described (44). RNA was also collected from the cartilage growth plates of these organ cultures in order to perform real-time PCR (see below).

3.3.3 Transforming growth factor alpha knockout mice

Tgfa null mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). All animals were bred and cared for in accordance with the University of Western Ontario's Animal Care and Use Guidelines. Heterozygous mice were crossed with each other so that litters with all three genotypes (wild type, heterozygous, homozygous null) could be obtained. PCR genotyping was performed using DNA from digested ear and/or tail clippings. Two separate genotyping programs were used to amplify both the wild type *Tgfa* allele and the neo-cassette found in the mutated alleles. Oligonucleotides TGFalphaA (5'-GACTAGCCTGGGCTACACAGTG-3') TGFalphaC (5'-

ACATGCTGGCTTCTCTTCCTGC-3'), NeoForward (5'-CTTGGGTGGAGA GGCTATTC-3') and NeoReverse (5'-AGGTGAGATGACAGGAGATC-3') were purchased from Sigma-Aldrich (Oakville, Ont., Canada) and all other genotyping reagents were purchased from Applied Biosystems Incorporated (Foster City, CA, USA). Animals were sacrificed by various methods depending on age (P0- decapitation, P10- intraperitoneal euthanal (pentobarbital sodium) injection, older animals- CO_2 chamber).

3.3.4 Skeletal preparations

Twenty-one day old (P21) and ten-week-old wild type, heterozygous, and *Tgfa* null littermates were sacrificed, eviscerated and full skeletons were stained with alizarin red and alcian blue as previously described (48). Three sets of littermates (each containing one wild type, one heterozygous, and one knockout mouse) were analyzed for each age group.

3.3.5 Histology

Newborn (P0), P21, and ten-week-old littermates were sacrificed. Tibiae only were isolated from P0 mice while tibiae and humeri were isolated from P21 and ten-week-old mice. Tibiae and humeri were chosen because they represent long bones from the fore and hind limbs, and also represent both proximal and distal bones. Tissues were fixed overnight in 4% paraformaldehyde (PFA) and decalcified as required in a 5% EDTA solution. Decalcification was not required for P0 tissues and was determined by a physical endpoint test in older tissues. Tissues were then processed, embedded in paraffin wax, and 5 μm thick sections were prepared by the Molecular Pathology Laboratory at the Robarts Research Institute (London, ON, Canada).

3.3.6 Safranin-O/fast green staining

Sections were dewaxed in xylene and rehydrated through a series of graded ethanols ending in water. Tissues were stained in 0.02% fast green for 25 minutes, dipped in 1% glacial acetic acid, then stained in 0.1% safranin-O for 7 minutes. Tissues were dehydrated and coverslips were mounted using a xylene-based mounting medium.

3.3.7 Bone length and growth plate zone measurements

Prior to decalcification, the lengths of tibiae and humeri were measured using a Zeiss Stemi DV4 Stereo microscope with eyepiece. The long bones of three sets of littermates were measured for P21 and ten-week-old mice and data were analyzed using one-way ANOVAs and Tukey's post-tests.

Pictures were taken of the growth plates of P0, P21, and ten–week-old littermates using a Retiga EX camera connected to a Leica DMRA2 microscope. Growth plate zones (resting, proliferating, hypertrophic) were measured by a blinded observer using OpenLab 4.0.4 software. The growth plate zones were distinguished by the unique morphology of residing chondrocytes (resting zone - small and round, proliferating zone - stacked and disc-like, hypertrophic zone - clearly enlarged), and the blinded observer had expertise in the arrangement and organization of the growth plate. Three sets of littermates were used for each age group and measurements were statistically analyzed using two-way ANOVAs with Bonferroni post-tests.

3.3.8 TRAP staining and immunohistochemistry

In order to assess osteoclast number, tartrate resistant acid phosphatase (TRAP) staining was performed on P21 tissues according to the manufacturer's protocol with some

modifications as previously described (43). TRAP staining was quantified in P21 tibiae by counting the number of TRAP-positive foci along the cartilage/bone junction. An area extending 1000 μm wide and 200 μm long (100 μm above and 100 μm below the junction) was outlined. All positive foci within this area were included. Counts from three independent trials were recorded and analyzed using one-way ANOVA with Tukey's post-tests. Immunohistochemistry was also carried out as previously described (40, 45, 48). Primary antibody incubation occurred overnight at 4^{o}C. Primary antibodies included anti-platelet endothelial cell adhesion molecule 1 (PECAM-1) antibody (sc-1506-R, Santa Cruz, Santa Cruz, CA, USA). Incubation with horseradish peroxidase-conjugated secondary antibody (sc-2004, Santa Cruz, Santa Cruz, CA, USA) was followed by colorimetric detection with the substrate diaminobenzidine (DAB). For all staining, three independent trials were used, and representative images are shown.

3.3.9 RNA isolation and real-time PCR

Growth plates were dissected from the ends of P0 tibiae and humeri with the aid of a Zeiss Stemi DV4 Stereo microscope as previously described (48). To avoid contamination, all surrounding connective tissue was removed and growth plates were rinsed in sterile Puck's Solution A. To ensure that the entire growth plate was collected, cartilage was removed with a scalpel just above the mineralized zone. Cartilage was clearly distinguishable from the grey area of mineralization. Alternatively, RNA was isolated from E15.5 tibia cartilage at the end of culture as described (21, 22). Samples were placed in QIAzol solution (Qiagen, Mississauga, ON, Canada), and RNA was isolated following the manufacturer's protocol. RNA was quantified and its quality was assessed before real-time PCR was carried out as described (48, 50). Analyses were

performed using the Applied Biosystems 7900HT Real-Time PCR system, the TaqMan® One-step Mastermix Kit, and commercially available probes (Applied Biosystems, Foster City, CA, USA). All samples were normalized to the housekeeping gene *Gapdh* and wild type controls using the delta-delta cycle threshold (ΔΔCT) method. Statistical analysis was carried out using paired t-tests.

3.3.10 Statistical analysis

All statistical analysis was performed using GraphPad Prism software Version 4.0 and graphs show mean values + standard error of the mean (SEM). A minimum of three independent groups was used in all experiments.

3.4 Results

3.4.1 Human recombinant TGFα does not affect growth of E15.5 tibiae in organ culture

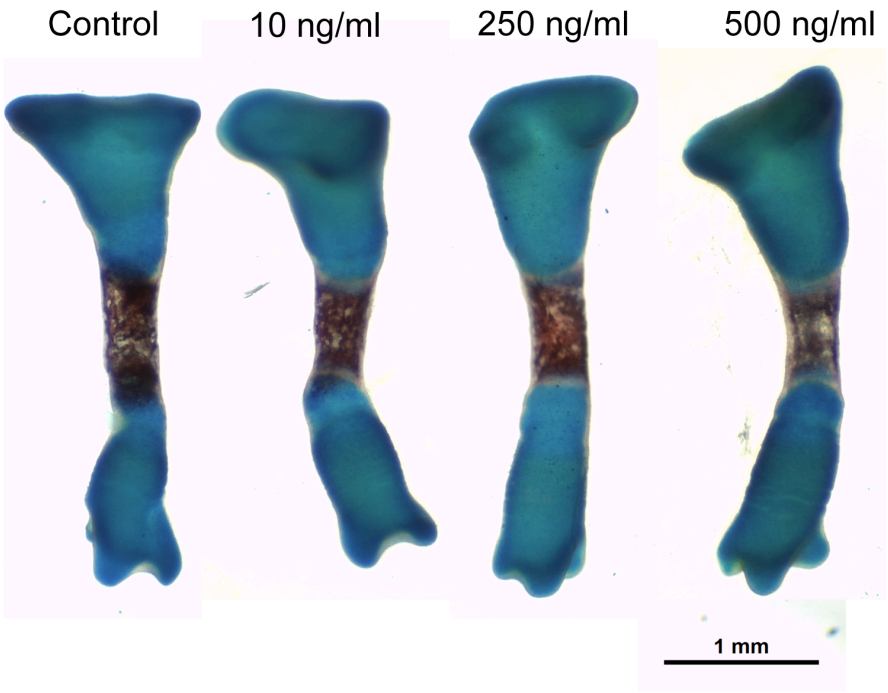
E15.5 mouse tibia organ cultures were treated with and without exogenous TGFα (0-1000 ng/ml) for six days. Representative images show control and treated tibiae (Figure 3.1A). Length measurements at the beginning and end of culture showed no change in overall growth over a range of TGFα concentrations (Figure 3.1B). This absence of effects suggests that TGFα is not a major regulator of chondrocyte proliferation or hypertrophy.

3.4.2 *Tgfa* null mice have shorter tibiae than their littermates

Full skeletons of male P21 wild type, heterozygous, and knockout littermates were stained with alizarin red and alcian blue (Figure 3.2A). No gross skeletal abnormalities were observed. However, mutant mice had significantly shorter tibiae than their wild type

Figure 3.1 Growth of TGFα-treated tibia organ cultures. Tibiae were isolated from E15.5 CD1 mice, grown in culture and treated with a range of TGFα concentrations. Whole tibiae were stained with alizarin red and alcian blue at the end of culture (A). Average growth over a six-day period was measured and is represented in millimetres +SEM (B). There is no change in growth with any of the concentrations used (n=4).

A



B

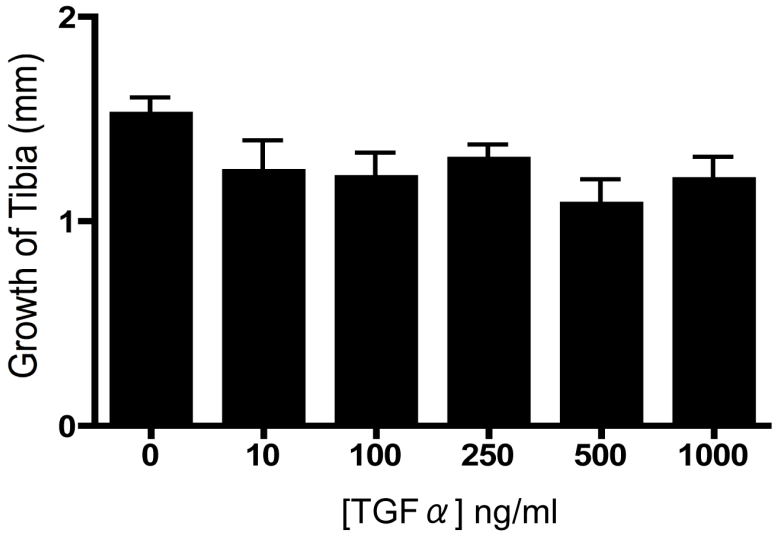


Figure 3.1 Growth of TGFα-treated tibia organ cultures.

Figure 3.2 *Tgfa* null mice have shorter tibiae than their littermates. P21 and ten-week-old mice were sacrificed and skeletons were stained with alizarin red and alcian blue (A, D). Mean length of P21 tibiae (B) and humeri (C) as well as ten-week-old tibiae (E) and humeri (F) + SEM is shown. P21 knockout mice have significantly shorter tibiae and humeri than their littermates, but no differences were seen at ten weeks of age. WT= wild type, Het= heterozygous, KO= knockout (n=4/group, *: $p<0.05$).

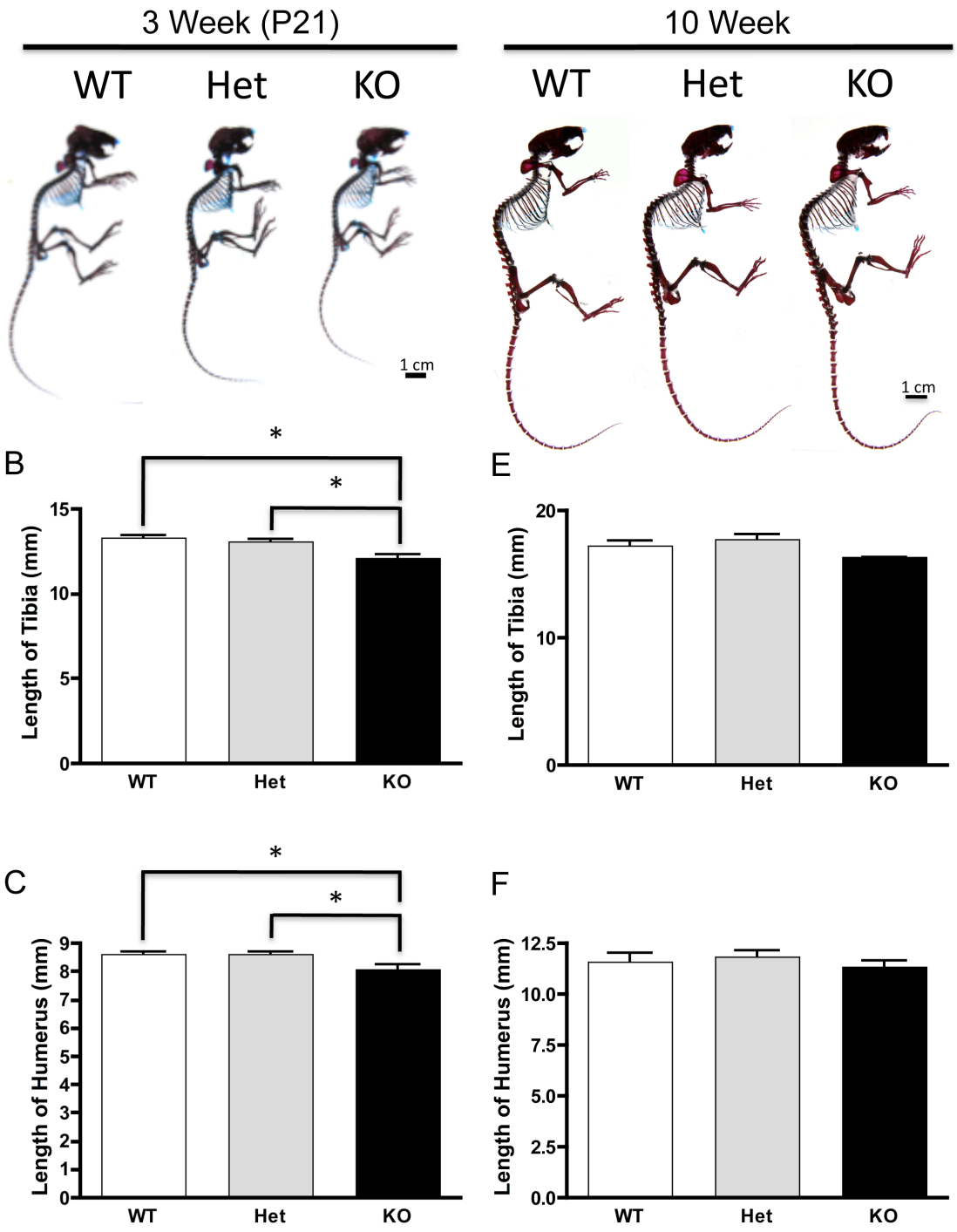


Figure 3.2 *Tgfa* null mice have shorter tibiae than their littermates.

(8.7% reduction) and heterozygous (7.3% reduction) littermates (Figure 3.2B). Mutant humeri were also found to be shorter than wild type and heterozygous littermates by about 6.4% (Figure 3.2C). To determine whether this phenotype was transient or persistent, we prepared skeletons and measured bones of ten-week-old male animals (Figure 3.2D). There were no significant differences in the length of tibiae or humeri at this time point (Figure 3.2E, F). Similar results were seen for female littermates (data not shown). With the exception of P21 females, body weight was not significantly different between genotypes (data not shown), suggesting that TGFα deficiency specifically affects skeletal growth.

3.4.3 *Tgfa* null mice have expanded hypertrophic zones

In order to assess growth plate organization, growth plates from P0, P21, and ten-week-old mice were stained with safranin-O and fast green. Figure 3.3 shows representative images and measurements from these animals. P0 and P21 *Tgfa* null mice have well organized growth plates with clearly expanded hypertrophic zones (Figure 3.3A). Measurement of growth plate zone lengths demonstrated that the hypertrophic zone, as well as the total growth plate, were significantly longer in *Tgfa* null mice when compared to their littermates, while length and organization of the resting and proliferating zones were unchanged (Figure 3.3B, 3.3C). In contrast, ten-week-old mice showed no differences in length of individual growth plate zones or the overall length and organization of the growth plate (Figure 3.3A, D). Similar findings were also found in female mice (data not shown).

Figure 3.3. *Tgfa* null mice have expanded hypertrophic zones. P0 and P21 and ten-week-old tibia growth plates were stained with safranin-O and fast green (A). Growth plate zones (RZ= resting zone, PZ = proliferating zone, HZ= hypertrophic zone) were measured for P0 (B), P21 (C), and ten-week-old (D) animals. Mean lengths +SEM are shown. P0 and P21 knockout mice have larger hypertrophic zones (labeled by double arrows) and larger growth plates overall than their littermates. No change in growth plate zones was observed at ten weeks. WT= wild type, Het= heterozygous, KO= knockout (n=4/group, *: $p<0.05$).

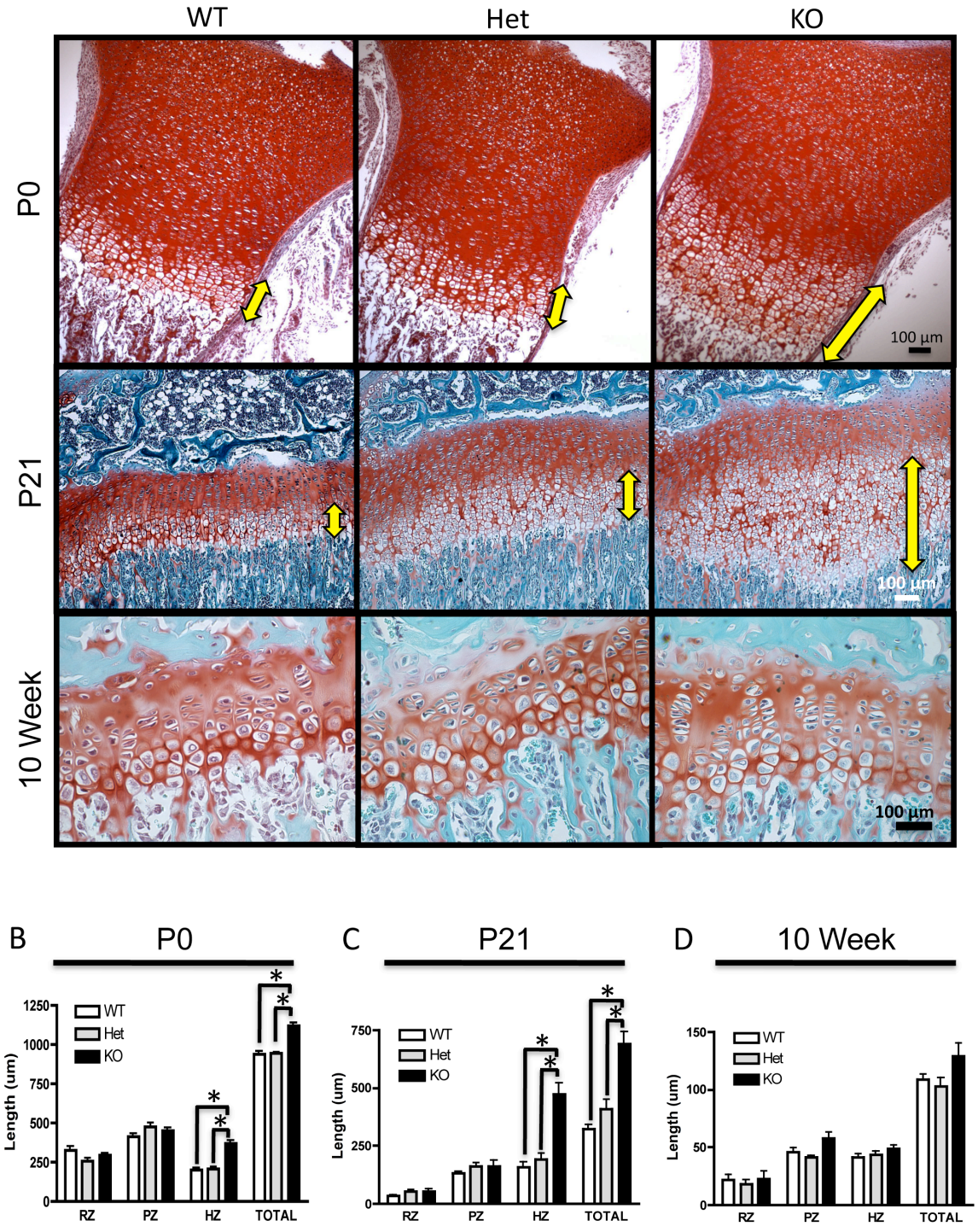


Figure 3.3 *Tgfa* null mice have expanded hypertrophic zones.

3.4.4 *Tgfa* null mice have fewer osteoclasts than their littermates

Our analysis of growth plate zones did not suggest effects of TGFα deficiency on the initiation of hypertrophy, suggesting that the larger hypertrophic zones in *Tgfa* null mice might be due to delayed resorption of cartilage. In order to assess osteoclast number, tartrate resistant acid phosphatase (TRAP) staining was performed on P21 tissues. TRAP staining was observed in early trabecular, cortical, and subchondral bone, and differences were observed near the growth plate/trabecular interface (Figure 3.4A). *Tgfa* null tissues have diminished TRAP staining compared to their wild type and heterozygous littermates (Figure 3.4A). The number of TRAP-positive foci was quantified along the cartilage/bone junction, and *Tgfa* null mice were found to have significantly fewer positive foci than their littermates (Figure 3.4B). Heterozygous mice also had significantly fewer TRAP-positive foci compared to wild type mice, indicating that TRAP expression may be dose dependent (Figure 3.4B). We also performed TRAP staining on P0 and ten day old (P10) tissues. While we found no major differences at P0, P10 patterns appeared similar to those of P21 showing less TRAP staining along the growth plate/trabecular bone border in mutant animals (Supplementary Figure 3.1).

3.4.5 RUNX2, MMP13, and RANKL gene expression are decreased in *Tgfa* null mice

To better understand the mechanisms at work in the mutant growth plate, we isolated RNA from cartilage of P0 litters and performed real-time PCR on a number of genes known to be involved in cartilage and/or bone development. In particular, we dissected the cartilage region giving rise to the secondary ossification center to examine genes involved in this process. We found that mRNA levels of a number of genes including *Sox9, Acan* (encoding aggrecan), *Col2a1* (type II collagen), *Col10a1* (type X collagen),

Figure 3.4 *Tgfa* null mice show reduced TRAP staining compared to their littermates. P21 tissues were stained for the osteoclast (OC) marker tartrate resistant acid phosphatase (TRAP, dark purple)(A). The number of TRAP-positive foci were then quantified along the cartilage/bone interface (B). There are fewer OCs along the cartilage growth plate/trabecular bone interface in knockout mice compared to their wild type and heterozygous littermates. As well, there are significantly less TRAP-positive foci in the heterozygous group compared to the wild type group. WT= wild type, Het= heterozygous, KO= knockout (n=3/group, * : $p<0.05$).

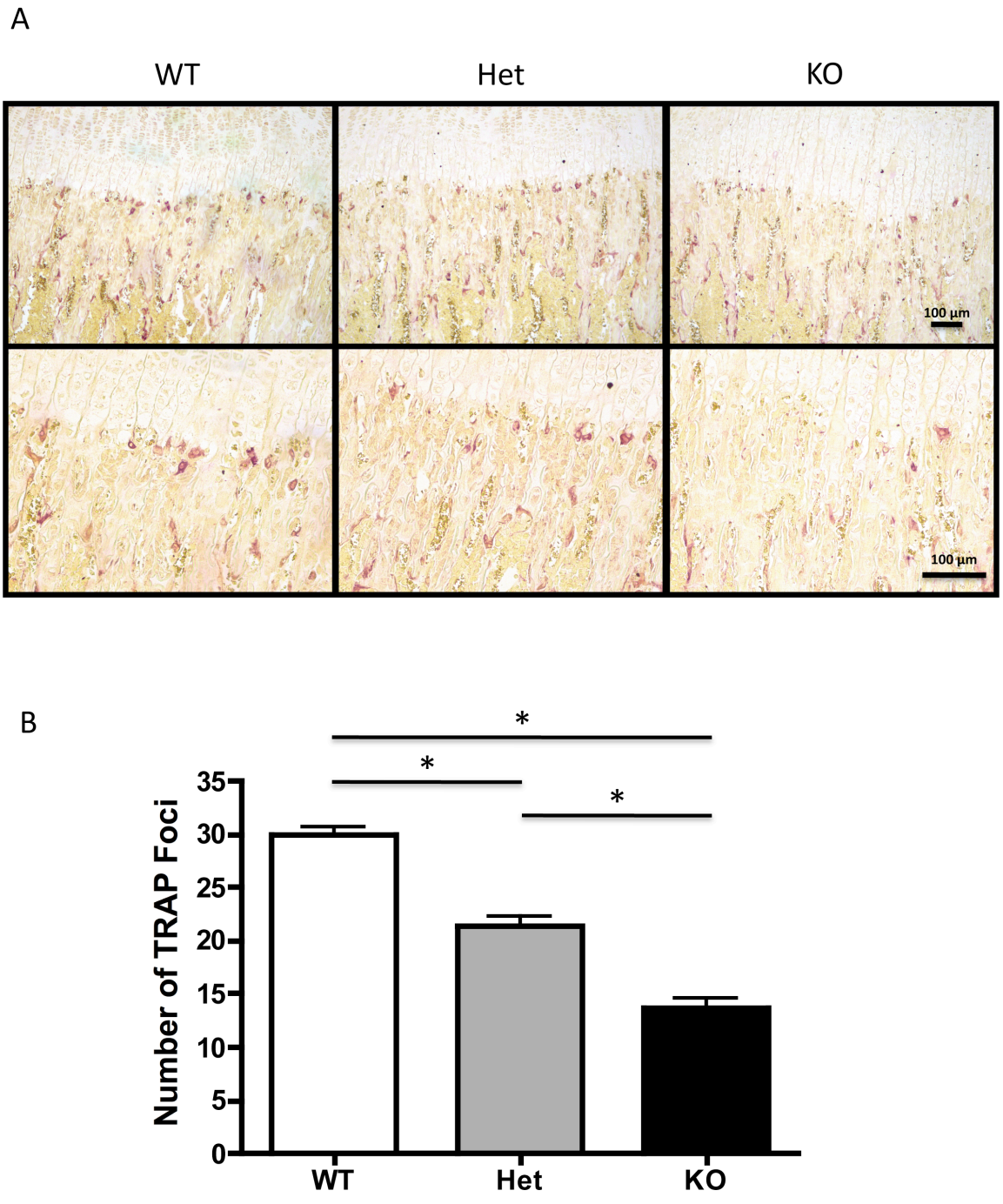


Figure 3.4 *Tgfa* null mice show reduced TRAP staining compared to their littermates.

Figure 3.5 *Runx2*, *Mmp13*, and *Tnfs11* expression are decreased in cartilage of *Tgfa* null mice. RNA was isolated from the growth plates of long bones of P0 mice and the expression of genes known to be involved in cartilage and bone physiology was assessed by real-time PCR. Many genes showed no change in expression levels between genotypes (A, B, C, E, G, I, J, K, L, M) while *Runx2*, *Mmp13*, and *Tnfs11* mRNA levels were all decreased in knockout animals (D, F, H). WT= wild type, KO = knockout (n=4/group, *: $p<0.05$).

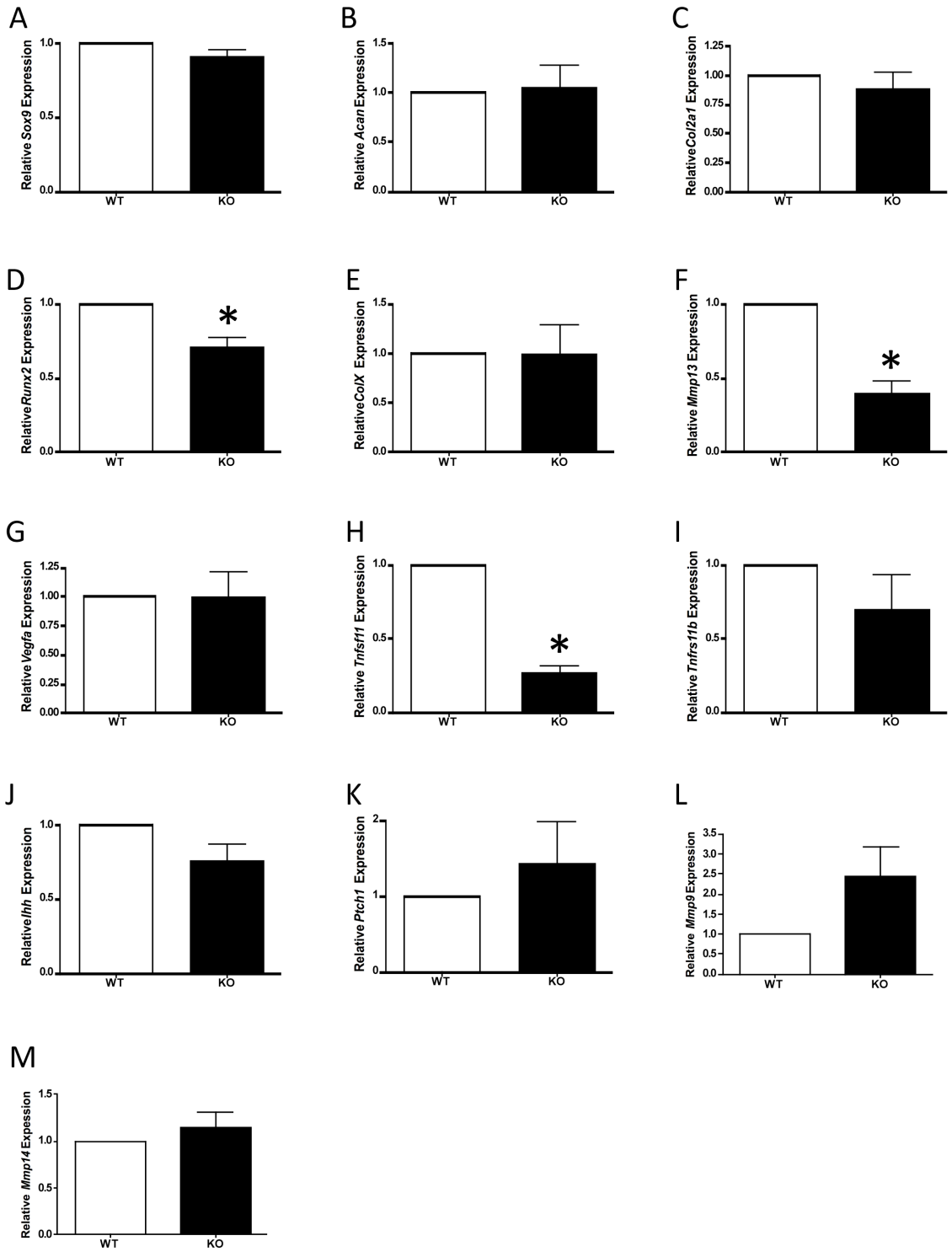


Figure 3.5 *Runx2*, *Mmp13*, and *Tnfs11* expression are decreased in cartilage of *Tgfa* null mice.

*Ihh (*Indian hedgehog*)*, *Tnfrs11b* (encoding osteoprotegerin, OPG), *Ptch1* (patched 1), *Vegfa* (vascular endothelial growth factor A), *Mmp9* (matrix metalloproteinase 9) and *Mmp14* (matrix metalloproteinase 14) remained unchanged between genotypes (Figure 3.5). However, expression levels of *Mmp13* (matrix metalloproteinase 13), *Tnfs11* (encoding receptor activator of nuclear factor kappa-B ligand, RANKL), and *Runx2* (runt-related transcription factor 2) were significantly reduced in mutant animals when compared to wild type littermates (Figure 3.5D, 3.5F, 3.5H).

3.4.6 E15.5 tibiae treated with TGFα show an increase in expression of late hypertrophic markers

To examine whether TGFα can increase expression of those genes reduced in TGFα-deficient cartilage, RNA was collected from the growth plates of E15.5 organ cultures treated with a range of TGFα concentrations. While no change in overall growth was noted (Figure 3.1), real-time PCR analyses reveal changes in the expression of hypertrophic markers. Notably, early hypertrophic markers including *p57* and *ColX* were decreased with TGFα treatment while late hypertrophic markers including *Mmp9*, *Mmp13*, and *Mmp14* were increased (Figure 3.6B-F). Furthermore, expression of the RANKL gene was increased with TGFα treatment (Figure 3.6G). A number of other genes (*Sox9*, *Acan*, *Col2a1*, *Aft3*, *Runx2*, *Vegfa*) showed no change in expression with TGFα treatment (Supplementary Figure 3.2).

3.4.7 *Tgfa* null mice experience delayed secondary ossification

To analyze whether secondary ossification would be affected in a manner similar to primary ossification, we examined the secondary ossification centers of P10 mice. *Tgfa* null mice showed delayed secondary ossification when compared to both wild type and

Figure 3.6 Early hypertrophic markers are decreased and late hypertrophic markers are increased in TGFα-treated E15.5 tibia organ cultures. Tibiae were isolated from E15.5 CD1 mice, grown in culture and treated with a range of TGFα concentrations. After 6 days of treatment, RNA was isolated from the cartilage of these tibiae (region depicted in A) and real-time PCR was performed (B-G). Data reveal decreases in *p57* and *ColX*, as well as increases in *Mmp9*, *Mmp13*, *Mmp14*, and *Tnfs11* transcript levels with treatment (n=3-4/group, *: $p<0.05$).

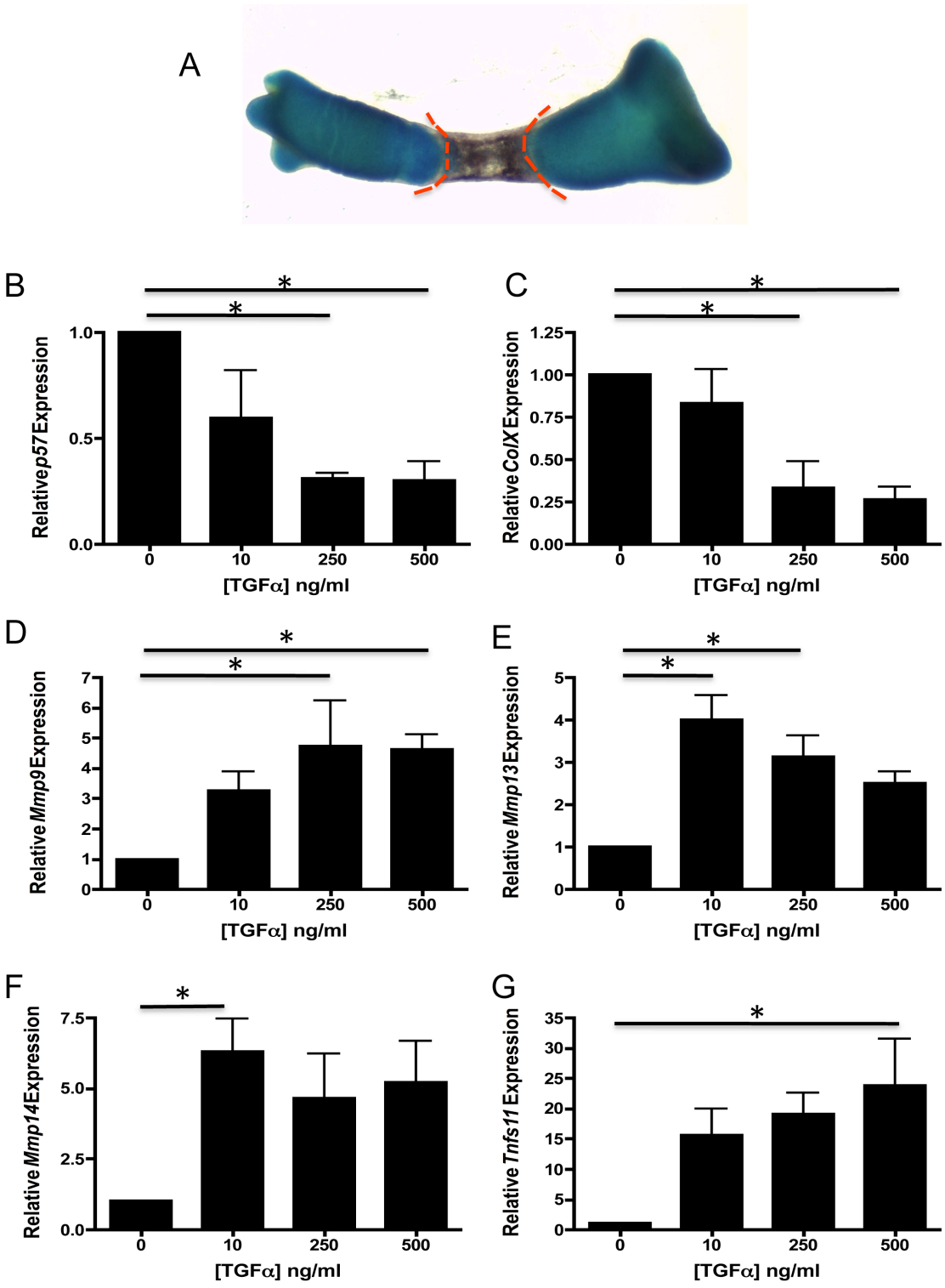


Figure 3.6 Early hypertrophic markers are decreased and late hypertrophic markers are increased in TGFα-treated E15.5 tibia organ cultures.

Figure 3.7 *Tgfa* null mice experience delayed secondary ossification and less PECAM-1 staining. P10 long bones were isolated and stained with safranin-O and fast green in order to observe secondary ossification centers (SOC). Knockout mice show the persistence of hypertrophic cells and an overall delay in secondary ossification compared to their littermates. Representative images show the SOCs of proximal humeri in the three genotypes. P10 long bones were stained with an anti-platelet endothelial cell adhesion molecule (PECAM-1) antibody in order to locate areas of vascularization. Knockout mice show less PECAM-1 staining than their littermates in the secondary ossification centers. WT= wild type, Het= heterozygous, KO= knockout (n=3).

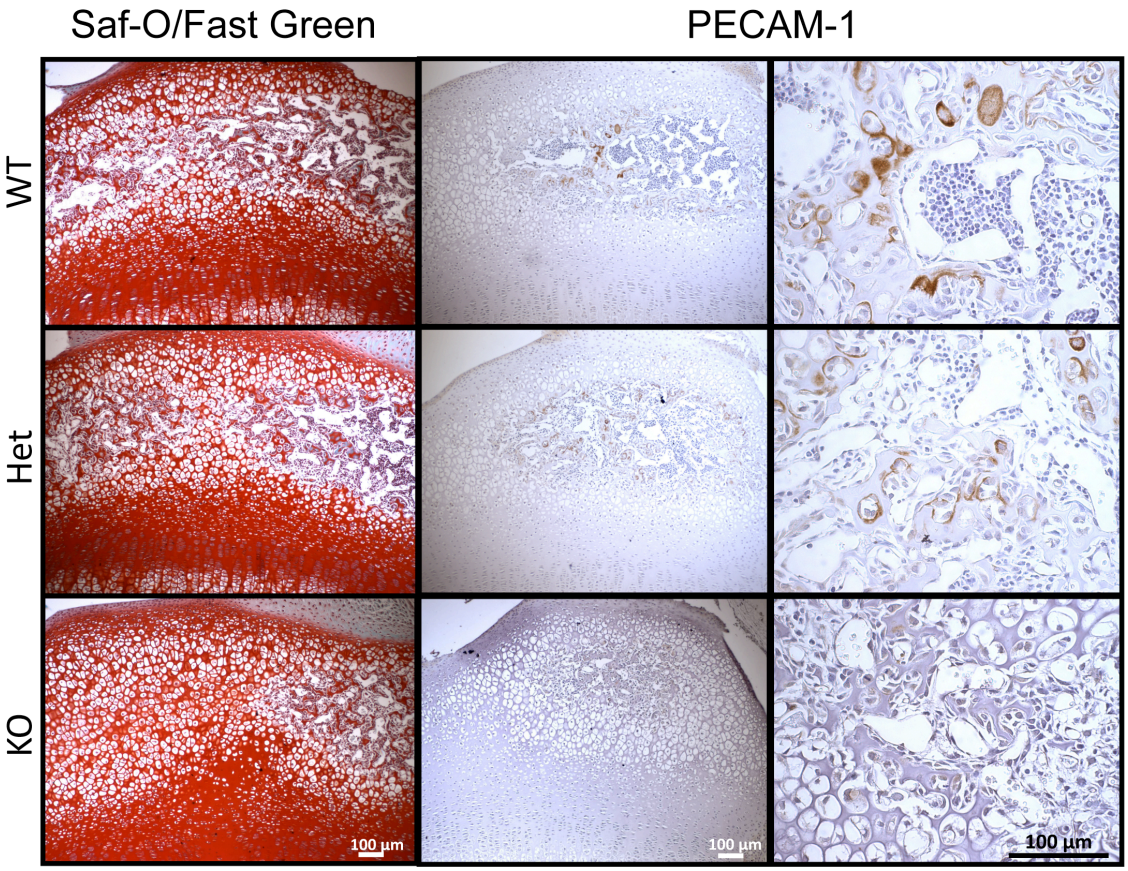


Figure 3.7 *Tgfa* null mice experience delayed secondary ossification and less PECAM-1 staining.

heterozygous littermates (Figure 3.7). To examine vascular invasion during the formation of secondary ossification centers, we performed IHC with an antibody against platelet endothelial cell adhesion molecule-1 (PECAM-1), a marker of endothelial cells. We found less PECAM-1 staining in the secondary ossification centers of knockout animals than in those of their littermates (Figure 3.7).

We also analyzed PECAM-1 staining at the growth plate/trabecular bone interface in P0, P10, and P21 tissues. PECAM-1 expression appeared similar among all genotypes at P0, but mutant tissues showed reduced PECAM-1 staining at P10 and P21 (Supplementary Figure 3.3).

3.5 Discussion

Our data show that *Tgfa* knockout (KO) mice have a transient growth plate and resulting skeletal growth phenotype that appears to resolve by ten weeks of age. Phenotypic changes include shorter tibiae and humeri, an enlarged hypertrophic zone and overall growth plate length, delayed primary and secondary ossification, and reduced osteoclast staining at the cartilage/bone junction.

The most dramatic aspect of this phenotype is the expanded hypertrophic zone. The appearance of the *Tgfa* null hypertrophic zone is similar to that of the *Egfr* knockout mouse previously described (49). However, *Egfr* mutants did not survive long after birth, and consequently analyses focused on embryonic and newborn mice only (49). While we studied post-natal time points, the resemblance between the two models is clear. Moreover, our collaborators recently showed a similar phenotype as described here in rats treated with an EGFR inhibitor and in mice with cartilage-specific inactivation of

EGFR (52). Since TGFα signals through the EGFR, it is likely that TGFα the major ligand responsible for regulating the terminal steps in endochondral ossification, specifically the replacement of hypertrophic cartilage by bone. However, the fact that the phenotype of *Tgfa* knockout mice is less severe than that of *Egfr* knockout mice suggests the involvement of additional EGFR ligands in this process.

TGFα effects in our organ culture experiments (that were performed at an early stage in skeletal development when mineralization had barely begun) are limited to markers of later stages of hypertrophy. This finding supports the model that TGFα does not directly control chondrocyte proliferation or early differentiation, but rather cartilage conversion to bone tissue. In contrast to our findings, classical experiments in mesenchymal micromass culture have indicated that TGFα is inhibitory to chondrogenesis (16). It is possible that TGFα effects may vary depending on the extent of chondrocyte differentiation at the start of the experiment (mesenchymal cells verses growth plate chondrocytes) as well as on the system of study (micromass cell culture versus in vivo and organ culture studies). Similarly, we recently showed mitogenic effects of TGFα on articular chondrocytes (4), while both our current study and results from our collaborators (52) suggest that TGFα does not affect growth plate chondrocyte proliferation.

In our studies, it appears that reduced osteoclast numbers are responsible for the persistence of hypertrophic cartilage. Our data show a clear decrease in the amount of TRAP staining at the cartilage/bone interface in mutant animals when compared to controls. The same localized pattern of staining was observed in rats treated with an EGFR inhibitor (52). Furthermore, RANKL gene expression is decreased in cartilage of

our mutant animals. RANKL is a key regulator of osteoclastogenesis and is required for bone and cartilage resorption (27). Chondrocyte-produced RANKL is able to induce osteoclast formation (46), and RANKL-deficient mice show an expanded hypertrophic zone similar to the one in our mice (25). A reduction in RANKL expression could explain why we see fewer osteoclasts in *Tgfa* null tissues. This mechanism is biologically plausible as previous studies have shown that TGFα signaling affects both osteoclastogenesis and bone resorption (17, 18, 42, 53).

In addition to the *Egfr* knockout mouse, *Mmp13* and *Mmp9* mutants display expanded hypertrophic zones, delayed vascularization, and delayed ossification (19, 41, 47). *Mmp13* null mice were also found to have abnormal TRAP and PECAM-1 staining (19). Depending on the particular mutant studied, the abnormal growth plate phenotype was either persistent or resolved over time (19, 41). These data are relevant since our *Tgfa* null growth plate chondrocytes show decreased *Mmp13* mRNA expression, in agreement with our previous osteoarthritis studies indicating that TGFα regulates *Mmp13* expression in articular chondrocytes (4, 5).

Runx2 was the third gene showing reduced expression in *Tgfa* knockout cartilage. *Runx2* regulates terminal chondrocyte differentiation in the cartilage growth plate (20, 24) and both *Mmp13* and the RANKL gene have been identified as downstream targets (11, 23). Thus, it seems plausible that *Runx2* is the primary target of TGFα in chondrocytes and mediates the effects of this growth factor on MMP13, RANKL and the removal of hypertrophic cartilage.

Interestingly, when we treated embryonic tibia organ cultures with exogenous TGFα, we saw results that complemented our *in vivo* data. Specifically, we saw increases in both MMP13 and RANKL gene expression with treatment, suggesting once again that TGFα controls factors involved in the conversion of cartilage to bone. In the organ culture studies, we also observed decreased expression of early hypertrophic markers (*p57* and *ColX*) and increased expression of late hypertrophic markers (*Mmp9*, *Mmp13*, and *Mmp14*). This could suggest that TGFα promotes transition from early hypertrophic chondrocytes to terminally differentiated cells. While ColX mRNA levels were not changed in newborn cartilage *in vivo*, this difference could be due to the stages of cartilage development analyzed in the two models or to differences between *in vivo* and *in vitro* models.

Our knockout animals also show delayed secondary ossification and reduced staining of the vascular marker PECAM-1. Both angiogenesis and matrix remodeling are crucial components of the terminal events of endochondral ossification (31), and EGFR signaling is known to have pro-angiogenic effects (2, 12, 35). TGFα specifically has been shown to increase the expression of vascular endothelial growth factor (VEGF) and other pro-angiogenic factors (angiopoietin-2, G-CSF, HGF, IL-6, IL-8 and PDGF-BB) in mesenchymal stem cells (9). Studies have shown that inactivation of VEGF leads to decreased resorption of terminal chondrocytes, similar to what we observe in our mutant mice (13). When examining gene expression, however, we did not see a change in *Vegfa* mRNA. This leads us to believe that the observed delay in vascularization may either be due to a reduction in other angiogenic factors or posttranscriptional suppression of VEGF expression, or it may be secondary to reduced matrix remodeling caused by decreases in

MMP13 and RANKL gene expression. Furthermore, it is known that MMP14 plays a crucial role in the development of the secondary ossification centre (15). Previous studies in our lab have shown that Akt-1 knockout mice also experience delayed secondary ossification that appears to be mediated by MMP14 (43). We have also previously shown that Akt-1 is a downstream target of TGFα (5). Since our tibia organ cultures indicate that TGFα regulates MMP14 expression, this could be another mechanism by which *Tgfa* mutant mice experience delayed secondary ossification.

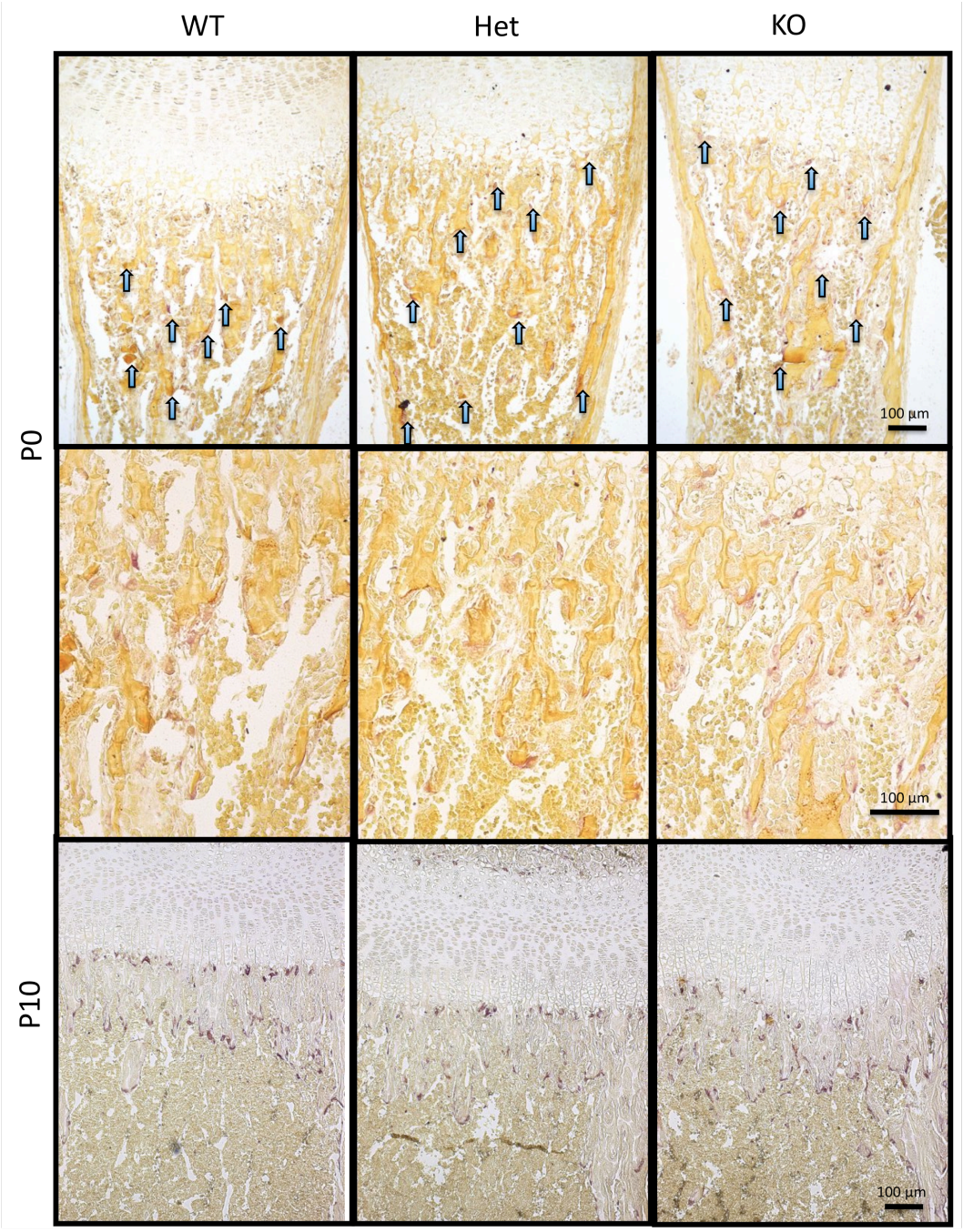
The transient nature of the phenotype observed here might be explained by a potential compensatory mechanism since there are multiple EGFR ligands: amphiregulin (AR), betacellulin (BTC), EGF, epigen (EPGN), epiregulin (EREG), heparin-binding EGF-like growth factor (HBEGF), and TGFα (14). The indication for compensation is strong since mice lacking multiple ligands can still have subtle phenotypes. For example, triple knockouts lacking EGF, AR, and TGFα were found to be healthy and fertile, but had problems with mammary gland development (30). The roles of several EGFR ligands have been investigated in bone physiology through in vivo transgenic or knockout models (8, 36, 37), and most of these models show subtle skeletal effects (reviewed by Schneider et. al. (38)). We examined the gene expression of AR, EGF, and HBEGF in our *Tgfa* wild type and knockout mice through real-time PCR (Supplementary Figure 3.4). Interestingly, we saw no changes in the expression levels of these ligands. There are several possible explanations for this. Perhaps no single ligand is essential for compensation, but rather several ligands contribute with overlapping roles. Alternatively, regulation of the other EGFR ligands might occur post-transcriptionally. Finally, the

RNA we studied was from P0 mice (for technical reasons). Compensation may occur gradually or at a later time point.

Another hypothesis to explain "catch-up growth" is delayed senescence of the growth plate (33, 34). Potentially, mice lacking TGFα could experience a temporary decreased growth rate that is later compensated by an increased duration of growth compared to control animals. Nevertheless, our data demonstrate a unique, non-redundant role of TGFα in endochondral ossification during early postnatal development.

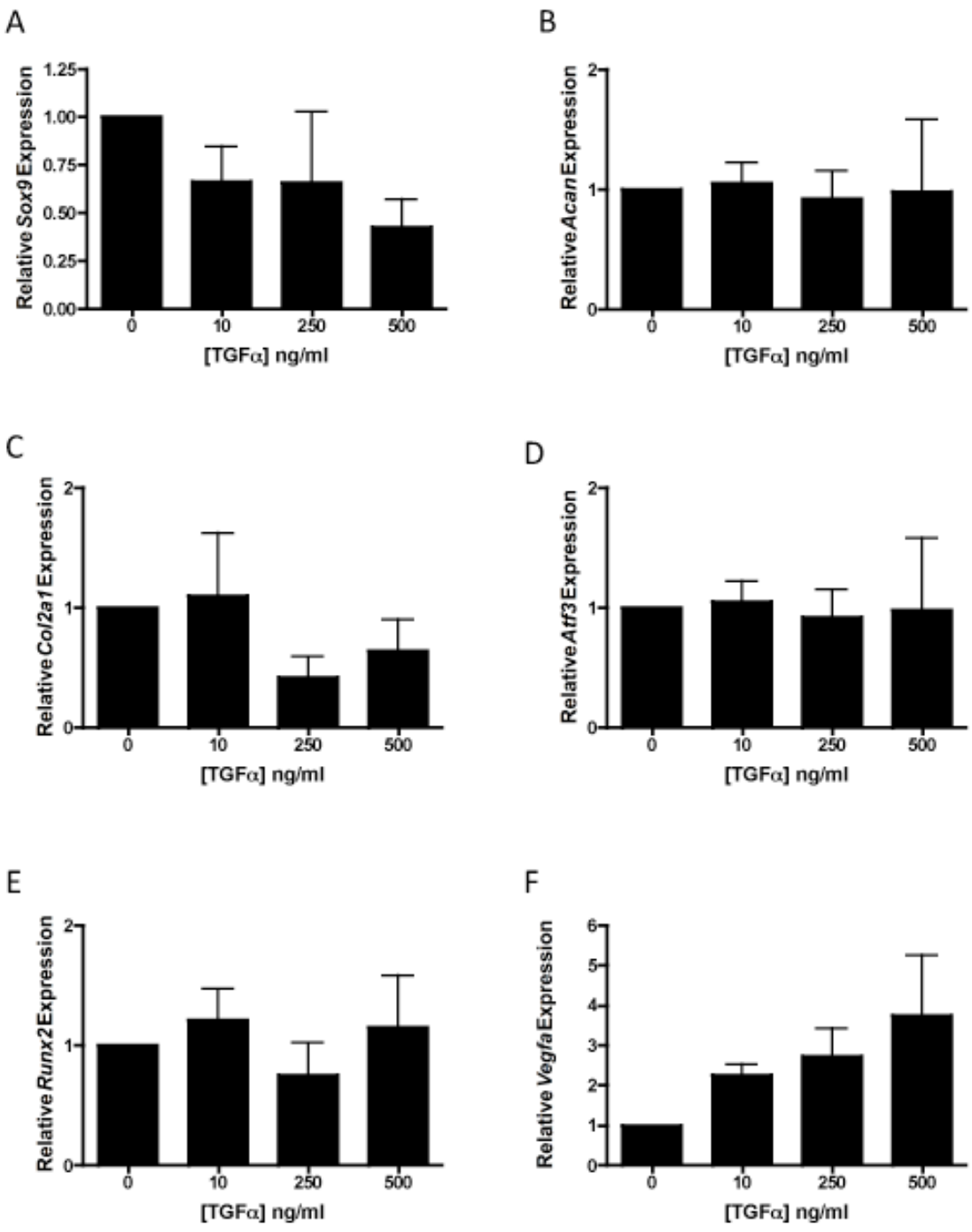
In conclusion, our data show that *Tgfa* knockout mice have a hitherto unrecognized growth plate phenotype. TGFα plays an important role in regulating endochondral ossification, osteoclast recruitment, and vascularization at the primary and secondary ossification center and appears to be the major ligand responsible for the observed roles of EGFR in endochondral ossification.

Supplementary Figure 3.1 *Tgfa* null mice show reduced TRAP staining along the cartilage/bone interface. P0 and P10 tissues were stained for the osteoclast (OC) marker tartrate resistant acid phosphatase (TRAP, dark purple). TRAP staining appears similar amongst various genotypes in P0 tissues and is located in discrete areas throughout the primary ossification centre (indicated by arrows). P10 knockout mice show reduced TRAP staining along the cartilage/bone interface when compared to their control littermates. WT= wild type, Het= heterozygous, KO= knockout (n=3).



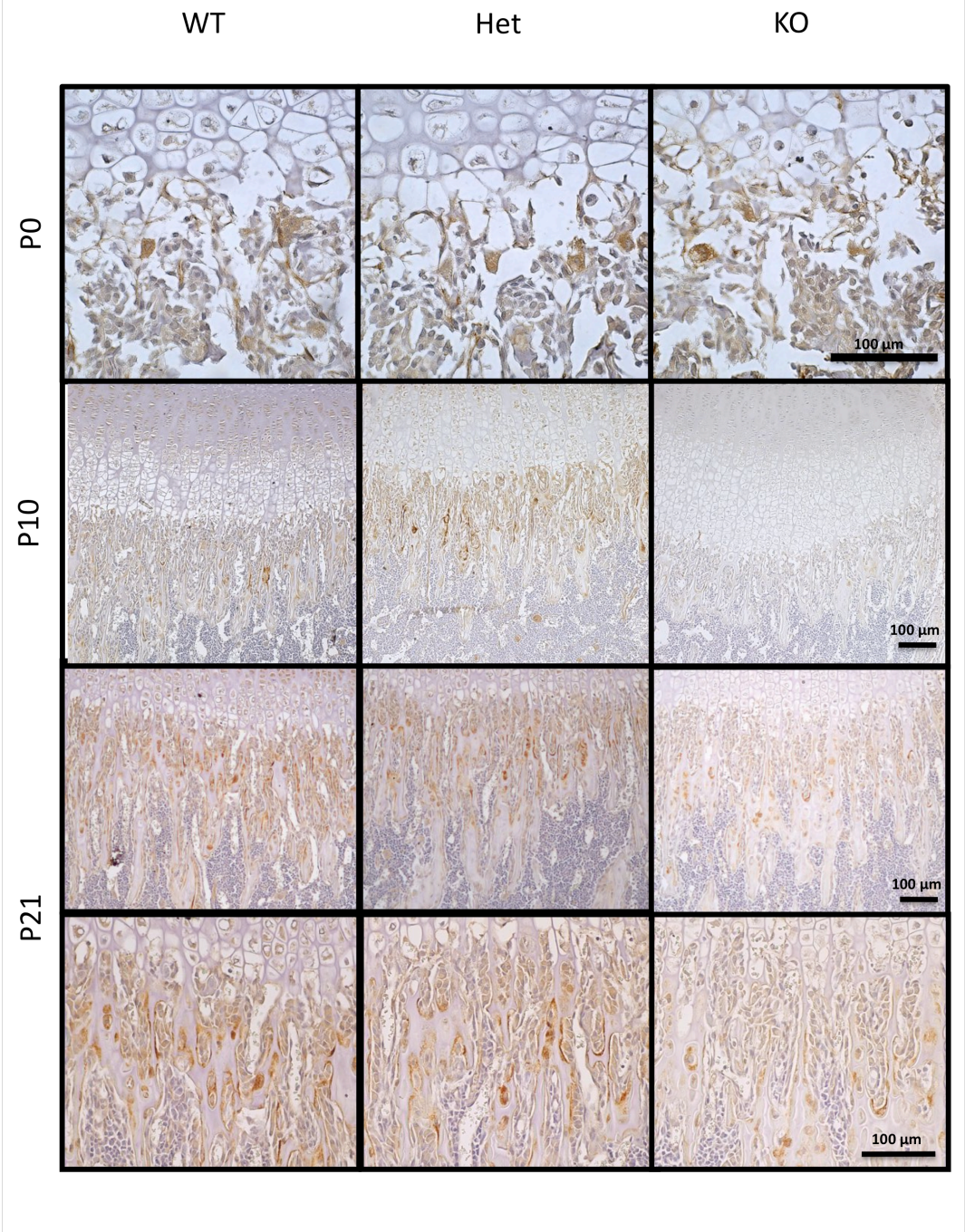
Supplementary 3.1 *Tgfa* null mice show reduced TRAP staining along the cartilage/bone interface.

Supplementary Figure 3.2 The expression of many genes remains unchanged in TGFα-treated E15.5 tibia organ cultures. Tibiae were isolated from E15.5 CD1 mice, grown in culture and treated with a range of TGFα concentrations. After 6 days of treatment, RNA was isolated from the cartilage growth plates of these tibiae and real-time PCR was performed. No changes in the expression of *Sox9*, *Acan*, *Col2a1*, *Atf3*, *Runx2*, or *Vegfa* were observed (n=3-4/group, *: $p<0.05$).



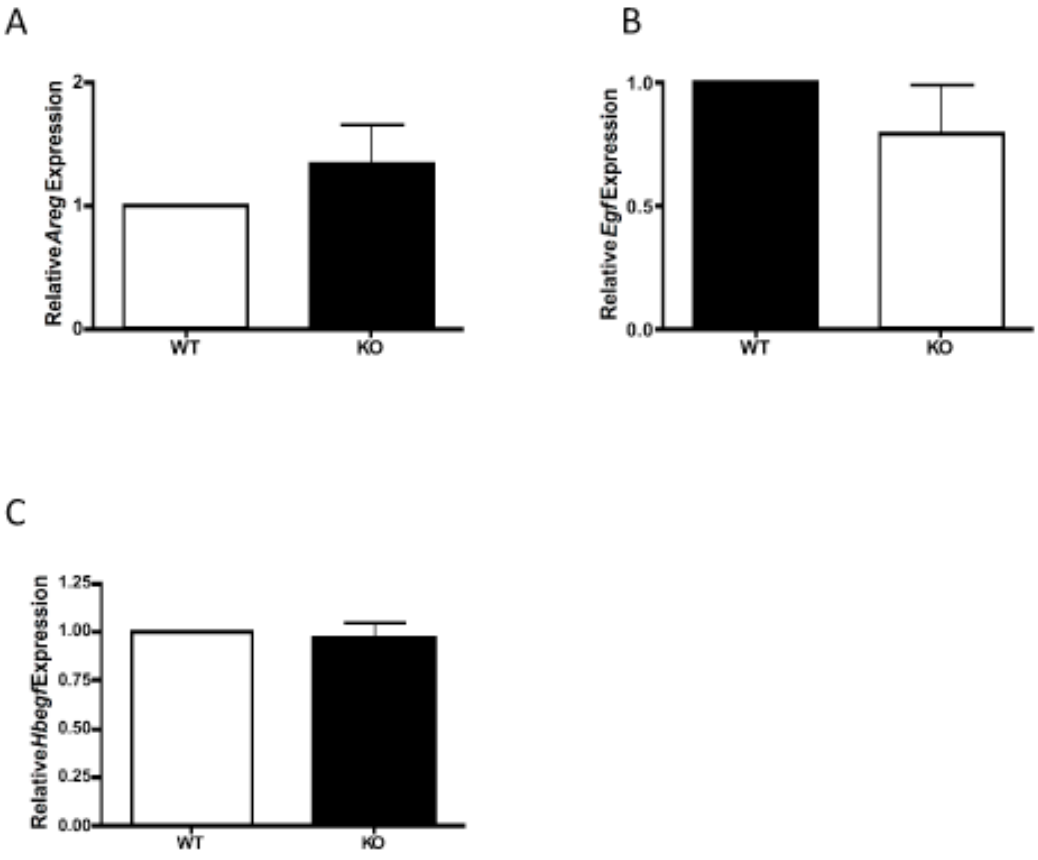
Supplementary 3.2 The expression of many genes remains unchanged in TGFα-treated E15.5 tibia organ cultures.

Supplementary Figure 3.3 *Tgfa* null mice show less PECAM-1 staining than their littermates at the cartilage/bone interface. P0, P10 and P21 long bones were stained with an anti-platelet endothelial cell adhesion molecule (PECAM-1) antibody in order to locate areas of vascularization. PECAM-1 expression appears similar in all P0 genotypes. P10 and P21 mutant mice show reduced PECAM-1 staining compared to their littermates along the cartilage/bone interface. WT= wild type, Het= heterozygous, KO= knockout (n=3).



Supplementary 3.3 *Tgfa* null mice express less PECAM-1 than their littermates at the cartilage/bone interface.

Supplementary Figure 3.4 *Tgfa* null mice do not show compensatory increases in transcript levels for the EGFR ligands *Areg*, *Egf*, and *Hbegf*. RNA was isolated from the growth plates of long bones of P0 mice and the expression of amphiregulin (*Areg*), epidermal growth factor (*Egf*), and heparin-binding EGF-like growth factor (*Hbegf*) was assessed by real-time PCR (A-C). There was no change in gene expression level for any of the EGFR ligands examined. WT= wild type, KO = knockout (n=4/group, *: $p<0.05$).



Supplementary 3.4 *Tgfa* null mice do not show compensatory increases in transcript levels for the EGFR ligands *Areg*, *Egf*, and *Hbegf*.

3.6 References

1. **Agoston H, Khan S, James CG, Gillespie JR, Serra R, Stanton LA, and Beier F.** C-type natriuretic peptide regulates endochondral bone growth through p38 MAP kinase-dependent and -independent pathways. *BMC Dev Biol* 7: 18, 2007.

2. **Akagi M, Kawaguchi M, Liu W, McCarty MF, Takeda A, Fan F, Stoeltzing O, Parikh AA, Jung YD, Bucana CD, Mansfield PF, Hicklin DJ, and Ellis LM.** Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. *Br J Cancer* 88: 796-802, 2003.

3. **Appleton CT, Pitelka V, Henry J, and Beier F.** Global analyses of gene expression in early experimental osteoarthritis. *Arthritis Rheum* 56: 1854-1868, 2007.

4. **Appleton CT, Usmani SE, Bernier SM, Aigner T, and Beier F.** Transforming growth factor alpha suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. *Arthritis Rheum* 56: 3693-3705, 2007.

5. **Appleton CT, Usmani SE, Mort JS, and Beier F.** Rho/ROCK and MEK/ERK activation by transforming growth factor-alpha induces articular cartilage degradation. *Lab Invest* 90: 20-30, 2010.

6. **Ballock RT and O'Keefe RJ.** The biology of the growth plate. *J Bone Joint Surg Am* 85-A: 715-726, 2003.

7. **Beier F.** Cell-cycle control and the cartilage growth plate. *J Cell Physiol* 202: 1-8, 2005.

8. **Chan SY and Wong RW.** Expression of epidermal growth factor in transgenic mice causes growth retardation. *J Biol Chem* 275: 38693-38698, 2000.

9. **De Luca A, Gallo M, Aldinucci D, Ribatti D, Lamura L, D'Alessio A, De Filippi R, Pinto A, and Normanno N.** The role of the EGFR ligand/receptor system in the secretion of angiogenic factors in mesenchymal stem cells. *J Cell Physiol*, 2010.

10. **Dealy CN, Scranton V, and Cheng HC.** Roles of transforming growth factor-alpha and epidermal growth factor in chick limb development. *Dev Biol* 202: 43-55, 1998.

11. **Geoffroy V, Kneissel M, Fournier B, Boyde A, and Matthias P.** High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage. *Mol Cell Biol* 22: 6222-6233, 2002.

12. **Gerber HP and Ferrara N.** Angiogenesis and bone growth. *Trends Cardiovasc Med* 10: 223-228, 2000.

13. **Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, and Ferrara N.** VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 5: 623-628, 1999.

14. **Harris RC, Chung E, and Coffey RJ.** EGF receptor ligands. *Exp Cell Res* 284: 2-13, 2003.

15. **Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM, and Birkedal-Hansen H.** MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 99: 81-92, 1999.

16. **Huang L, Solursh M, and Sandra A.** The role of transforming growth factor alpha in rat craniofacial development and chondrogenesis. *J Anat* 189 (Pt 1): 73-86, 1996.

17. **Ibbotson KJ, Harrod J, Gowen M, D'Souza S, Smith DD, Winkler ME, Derynck R, and Mundy GR.** Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. *Proc Natl Acad Sci U S A* 83: 2228-2232, 1986.

18. **Ibbotson KJ, Twardzik DR, D'Souza SM, Hargreaves WR, Todaro GJ, and Mundy GR.** Stimulation of bone resorption in vitro by synthetic transforming growth factor-alpha. *Science* 228: 1007-1009, 1985.

19. **Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, and Krane SM.** Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc Natl Acad Sci U S A* 101: 17192-17197, 2004.

20. **Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, Sato M, Yamagiwa H, Kimura T, Yasui N, Ochi T, Endo N, Kitamura Y, Kishimoto T, and Komori T.** Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Dev Dyn* 214: 279-290, 1999.

21. **James CG, Stanton LA, Agoston H, Ulici V, Underhill TM, and Beier F.** Genome-wide analyses of gene expression during mouse endochondral ossification. *PLoS One* 5: e8693.

22. **James CG, Woods A, Underhill TM, and Beier F.** The transcription factor ATF3 is upregulated during chondrocyte differentiation and represses cyclin D1 and A gene transcription. *BMC Mol Biol* 7: 30, 2006.

23. **Jimenez MJ, Balbin M, Lopez JM, Alvarez J, Komori T, and Lopez-Otin C.** Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. *Mol Cell Biol* 19: 4431-4442, 1999.

24. **Kim IS, Otto F, Zabel B, and Mundlos S.** Regulation of chondrocyte differentiation by Cbfa1. *Mech Dev* 80: 159-170, 1999.

25. **Kim N, Odgren PR, Kim DK, Marks SC, Jr., and Choi Y.** Diverse roles of the tumor necrosis factor family member TRANCE in skeletal physiology revealed by TRANCE deficiency and partial rescue by a lymphocyte-expressed TRANCE transgene. *Proc Natl Acad Sci U S A* 97: 10905-10910, 2000.

26. **Kobayashi T and Kronenberg H.** Minireview: transcriptional regulation in development of bone. *Endocrinology* 146: 1012-1017, 2005.

27. **Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, and Penninger JM.** OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397: 315-323, 1999.

28. **Kronenberg HM.** Developmental regulation of the growth plate. *Nature* 423: 332-336, 2003.

29. **Kumar V, Bustin SA, and McKay IA.** Transforming growth factor alpha. *Cell Biol Int* 19: 373-388, 1995.

30. **Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, and Lee DC.** Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 126: 2739-2750, 1999.

31. **Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, and Mirams M.** Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int J Biochem Cell Biol* 40: 46-62, 2008.

32. **Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, and Dunn AR.** Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 73: 249-261, 1993.

33. **Nilsson O and Baron J.** Fundamental limits on longitudinal bone growth: growth plate senescence and epiphyseal fusion. *Trends Endocrinol Metab* 15: 370-374, 2004.

34. **Nilsson O and Baron J.** Impact of growth plate senescence on catch-up growth and epiphyseal fusion. *Pediatr Nephrol* 20: 319-322, 2005.

35. **Perrotte P, Matsumoto T, Inoue K, Kuniyasu H, Eve BY, Hicklin DJ, Radinsky R, and Dinney CP.** Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 5: 257-265, 1999.

36. **Qin L, Tamasi J, Raggatt L, Li X, Feyen JH, Lee DC, Dicicco-Bloom E, and Partridge NC.** Amphiregulin is a novel growth factor involved in normal bone development and in the cellular response to parathyroid hormone stimulation. *J Biol Chem* 280: 3974-3981, 2005.

37. **Schneider MR, Dahlhoff M, Herbach N, Renner-Mueller I, Dalke C, Puk O, Graw J, Wanke R, and Wolf E.** Betacellulin overexpression in transgenic mice causes disproportionate growth, pulmonary hemorrhage syndrome, and complex eye pathology. *Endocrinology* 146: 5237-5246, 2005.

38. **Schneider MR, Sibilia M, and Erben RG.** The EGFR network in bone biology and pathology. *Trends Endocrinol Metab* 20: 517-524, 2009.

39. **Sibilia M, Wagner B, Hoebertz A, Elliott C, Marino S, Jochum W, and Wagner EF.** Mice humanised for the EGF receptor display hypomorphic phenotypes in skin, bone and heart. *Development* 130: 4515-4525, 2003.

40. **Solomon LA, Li JR, Berube NG, and Beier F.** Loss of ATRX in chondrocytes has minimal effects on skeletal development. *PLoS One* 4: e7106, 2009.

41. **Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, and Werb Z.** Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* 131: 5883-5895, 2004.

42. **Takahashi N, MacDonald BR, Hon J, Winkler ME, Derynck R, Mundy GR, and Roodman GD.** Recombinant human transforming growth factor-alpha stimulates the formation of osteoclast-like cells in long-term human marrow cultures. *J Clin Invest* 78: 894-898, 1986.

43. **Ulici V, Hoenselaar KD, Agoston H, McErlain DD, Umoh J, Chakrabarti S, Holdsworth DW, and Beier F.** The role of Akt1 in terminal stages of endochondral bone formation: angiogenesis and ossification. *Bone* 45: 1133-1145, 2009.

44. **Ulici V, Hoenselaar KD, Gillespie JR, and Beier F.** The PI3K pathway regulates endochondral bone growth through control of hypertrophic chondrocyte differentiation. *BMC Dev Biol* 8: 40, 2008.

45. **Ulici V, James CG, Hoenselaar KD, and Beier F.** Regulation of gene expression by PI3K in mouse growth plate chondrocytes. *PLoS One* 5: e8866, 2010.

46. **Usui M, Xing L, Drissi H, Zuscik M, O'Keefe R, Chen D, and Boyce BF.** Murine and chicken chondrocytes regulate osteoclastogenesis by producing RANKL in response to BMP2. *J Bone Miner Res* 23: 314-325, 2008.

47. **Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, and Werb Z.** MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 93: 411-422, 1998.

48. **Wang G, Woods A, Agoston H, Ulici V, Glogauer M, and Beier F.** Genetic ablation of Rac1 in cartilage results in chondrodysplasia. *Dev Biol* 306: 612-623, 2007.

49. **Wang K, Yamamoto H, Chin JR, Werb Z, and Vu TH.** Epidermal growth factor receptor-deficient mice have delayed primary endochondral ossification because of defective osteoclast recruitment. *J Biol Chem* 279: 53848-53856, 2004.

50. **Yan Q, Feng Q, and Beier F.** Endothelial nitric oxide synthase deficiency in mice results in reduced chondrocyte proliferation and endochondral bone growth. *Arthritis Rheum* 62: 2013-2022, 2012.

51. **Yoon YM, Oh CD, Kim DY, Lee YS, Park JW, Huh TL, Kang SS, and Chun JS.** Epidermal growth factor negatively regulates chondrogenesis of mesenchymal cells by modulating the protein kinase C-alpha, Erk-1, and p38 MAPK signaling pathways. *J Biol Chem* 275: 12353-12359, 2000.

52. **Zhang X, Siclari VA, Lan S, Zhu J, Koyama E, Dupuis HL, Enomoto-Iwamoto M, Beier F, and Qin L.** The critical role of the epidermal growth factor receptor in endochondral ossification. *J Bone Miner Res* 26: 2622-2633, 2011.

53. **Zhu J, Jia X, Xiao G, Kang Y, Partridge NC, and Qin L.** EGF-like ligands stimulate osteoclastogenesis by regulating expression of osteoclast regulatory factors by osteoblasts: implications for osteolytic bone metastases. *J Biol Chem* 282: 26656-26664, 2007.

Chapter 4

4 TGFα null mice experience delayed progression of osteoarthritis in a surgical disease model

Figures 4.2, 4.3, and 4.4 in this chapter will contribute to an original co-first author research article to be submitted for publication:

Appleton CTG and Usmani SE, Pest MA, Pitelka V, Ulici V, Welch I, Hill TL, Mort J, and Beier F. The TGFα-CCL2 axis is a target for treatment of osteoarthritis. To be submitted to *J Clin Invest,* 2012.

4.1 Abstract

We have previously identified transforming growth factor alpha (TGFα) as a novel growth factor involved in cartilage degeneration during the disease process of osteoarthritis (OA). *In vitro* experiments show that TGFα treatment induces an OA-like phenotype in articular chondrocytes, characterized by inhibition of matrix synthesis and promotion of catabolic factor expression. In order to better understand and validate TGFα's potential as a novel therapeutic target, we employed two *in vivo* models of the disease: a post-traumatic destabilization model and a spontaneous aging model. Ten-week-old male *Tgfa* null mice and their heterozygous (control) littermates underwent surgery resulting in destabilization of the medial meniscus (DMM). Disease progression was assessed histologically at both seven and fourteen weeks post-surgery using the Osteoarthritis Research Society International (OARSI) scoring system. Six-month-old mice also underwent the same DMM procedure and were assessed at seven weeks post-surgery. As well, spontaneous disease progression was analyzed in eighteen-month-old *Tgfa* null and heterozygous mice. We found that young, ten-week old knockout mice

were protected from OA progression at both seven and fourteen weeks post-surgery with statistically lower OARSI scores than their control littermates. When DMM was performed on six-month old animals however, no protection was seen. As well, no differences were observed between genotypes in the spontaneous again model.

In conclusion, young *Tgfa* null mice are protected from OA progression in the DMM model, while older mice are not. In addition, *Tgfa* null mice are equally susceptible to spontaneous OA development. Thus, TGFα might be a valuable therapeutic target in post-traumatic forms of OA, however its role in idiopathic disease less clear.

4.2 Introduction

Osteoarthritis (OA) is the most prevalent joint disease and the most common cause of physical disability in western society (22, 39). Patients with OA experience joint pain and impaired function that may ultimately affect their ability to work and quality of life (38, 43). Currently there are no treatment options that prevent, cure, or alter the disease course. Rather, therapeutic options are limited to symptom management (47). The financial consequences of managing OA are immense and relate to symptomatic treatment, joint replacement surgeries, and work lost to disability (9, 45). Current estimates suggest that developed nations spend approximately one to two percent of their gross domestic product on OA and other rheumatic diseases (20). Furthermore, due to high rates of obesity and the aging population, the incidence of OA is only expected to rise in the coming years (22). Consequently the identification of good therapeutic targets for the development of disease modifying osteoarthritic drugs (DMOADs) is urgently needed.

The defining feature of OA is the degeneration of articular cartilage – the tissue that covers the ends of diarthrodial joints and enables their smooth, wear-resistant movements. Articular cartilage consists of an extracellular matrix rich in type II collagen and proteoglycans and is designed to absorb and disperse the forces experienced during joint loading (2). The extracellular matrix is sparsely populated by chondrocytes and these cells are responsible for both synthesizing the matrix and regulating its turnover (35). A number of growth factors, cytokines, and catabolic enzymes are involved in normal cartilage metabolism as well as in OA (33). As of yet however, none of the drugs aimed at these therapeutic targets has been successful in preventing, stopping or reversing the disease process in clinical trials (37).

While the exact etiology of OA remains unclear, numerous risk factors have been identified (10, 49). Two of the most significant are joint injury and advanced age. It has been well established that a history of joint injury is a major risk factor for the development of OA (34). The causes of post-traumatic OA are vast but may involve intra-articular fractures, ligament tears, meniscal injuries, and even cartilage micro-trauma from joint impact (3). In the United States, meniscal injuries represent the highest proportion of all intra-articular injuries (30). The menisci are crescent-shaped fibrocartilagenous tissues found between the femur and the tibia in the medial and lateral compartments of the knee joint (13). They help nourish and lubricate the articular cartilage, act as shock absorbers, and transmit over half of the total axial load applied to the knee joint (30). Studies have indicated that individuals with ligamentous or meniscal injuries increase their risk of developing OA by ten-fold, and meniscal damage is almost always present in patients with radiographic evidence of OA (3, 13).

In addition to injury, advancing age is another major risk factor for OA. It is unusual to find individuals over the age of sixty-five to seventy without radiographic evidence of OA (26). Several theories have been developed to describe the link between aging and OA, although there is still a lack of consensus in this area. Perhaps the simplest theory is that OA is a "wear and tear" disease brought about by repetitive loading and an accumulation of damage over time (1). This theory, however, does not explain why radiographic signs are completely absent in some elderly individuals (18, 27). Other theories relate to specific age-related changes observed in the articular cartilage matrix and chondrocytes themselves. For example, aged cartilage undergoes more collagen cross-linking, thus making it stiffer and perhaps more susceptible to mechanical injury (25). As well, decreases in aggrecan deposition are seen with age, thus altering the cartilage's ability to deal with compressive forces (1). Aging also brings about various changes within the chondrocytes themselves including decreased responsiveness to anabolic cytokines such as transforming growth factor beta (TGFβ) and insulin-like growth factor-1 (IGF-1), and increased expression of reactive oxygen species (ROS) (26). Finally, remaining theories relate to aging and chondrocyte senescence, autophagy, and apoptosis (1, 25, 26).

In order to better understand the molecular mechanisms involved in OA, our lab recently studied a rodent anterior cruciate ligament (ACL) transection model to identify genes involved in cartilage degeneration (6). We identified transforming growth factor alpha (TGFα) as a novel growth factor involved in OA as its gene expression was increased in the disease state. Subsequent *in vitro* experiments showed that TGFα treatment resulted in a loss of chondrocyte phenotype: we observed decreases in anabolic factors including

type II collagen and aggrecan and increases in catabolic factors such as matrix metalloproteinase 13 (MMP13) (7). In this study, we wanted to determine the role of TGFα in the progression of OA *in vivo* and thus employed a post-traumatic OA model in the *Tgfa* knockout mouse. The OA model we used was the destabilization of medial meniscus (DMM) model which provides a relatively slowly progressing form of the disease that may have more relevance to the human disease course (16). We also observed spontaneous development of OA in aging *Tgfa* null mice. Since our previous studies suggested an overall catabolic effect of TGFα on chondrocytes, we hypothesized that *Tgfa* null mice would experience delayed OA progression in both the DMM model and in spontaneous disease development.

4.3 Methods

4.3.1 Transforming growth factor alpha null mice

Tgfa null mice in a C57BL/6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) (31). All animals were bred and cared for in accordance with the University of Western Ontario's Animal Care and Use Guidelines. DNA from digested ear and/or tail clippings was used in PCR genotyping. Two separate genotyping programs were used to amplify both the wild type *Tgfa* allele and the neo-cassette found in the mutated alleles. Oligonucleotides TGFalphaA (5'-GACTAGCCTGGGCTACACAGTG-3') TGFalphaC (5'-ACATGCTGGCTTCTCTTCCTGC-3'), NeoForward (5'-CTTGGGTGGAGAGGCT ATTC-3') and NeoReverse (5'-AGGTGAGATGACAGGAGATC-3') were purchased from Sigma-Aldrich (Oakville, Ont., Canada) and all other genotyping reagents were purchased from Applied Biosystems Incorporated (Foster City, CA, USA). All direct

comparisons were made between heterozygote and homozygote mutant siblings with at least four littermate pairs per time point per parameter examined.

4.3.2 Destabilization of medial meniscus (DMM) model

To study OA in *Tgfa* mutant animals, we employed the well-established surgical destabilization of medial meniscus (DMM) model (16). In this model, the medial meniscotibial ligament (which anchors the medial meniscus to the tibial plateau) is cleaved, resulting in joint destabilization. DMM was performed on the left knee joint of ten-week old male mice as previously described (44). Both *Tgfa* knockout mice and their heterozygous littermates were used in our studies. Heterozygous mice served as controls as previous studies have shown that *Tgfa* heterozygous mutants are phenotypically indistinguishable from their wild type littermates (31). Sham surgeries consisting of an incision to the left knee were also performed on both genotypes. A veterinarian and veterinarian technician with previous experience in the DMM procedure performed all surgeries. Mice were housed individually after surgeries. At seven and fourteen weeks post-surgery, mice were sacrificed and their knee joints were isolated for histology.

To examine the effects of age and injury together, we also performed DMM surgeries as described above in six-month-old mice, and assessed disease progression at seven weeks post-surgery.

4.3.3 Spontaneous osteoarthritis

To study the spontaneous development of OA, male and female *Tgfa* null mice were housed with their heterozygous littermates until eighteen months of age. Several male pairs were separated throughout this time course due to fighting. At eighteen months of

age, mice were sacrificed and their knee, ankle and elbow joints were isolated and prepared for histology.

4.3.4 Mouse tissue processing and histology

Tissues were fixed overnight in 4% PFA, and decalcified in 5% EDTA in PBS. Decalcification was determined by physical end-point test. Tissues were then processed, embedded in paraffin wax, and 5 μm thick serial sections were cut in the sagittal plane starting from the medial joint compartment. Previous studies have shown that the most severe damage in both DMM and spontaneous models of OA occur in the medial compartment (28, 32).

4.3.5 Safranin-O/fast green staining

Sections were dewaxed in xylene and rehydrated through a series of graded ethanols ending in water. Tissues were then stained in 0.02% fast green for 25 minutes, dipped in 1% glacial acetic acid, then stained in 0.1% safranin-O for 7 minutes. Tissues were dehydrated and mounted using a xylene-based mounting medium.

4.3.6 Immunohistochemistry

Immunohistochemistry was performed using primary antibodies against MMP13 (Abcam, San Francisco, CA) and type II collagen neoepitopes exposed when type II collagen is cleaved by MMP13 (8). Sections were first dewaxed in xylene, and rehydrated through a series of graded ethanol solutions ending in water. Antigen retrieval was performed in 10 mM sodium citrate at 95^{o}C for fifteen minutes. Sections were blocked in 5% goat serum, then incubated with primary antibodies overnight. Lastly, tissues were incubated in HRP-conjugated secondary antibody and visualized with the

substrate DAB (brown precipitate). All tissues were visualized using a Leica DM Series inverted fluorescence/light microscope (Leica Microsystems, Richmond Hill, ON, Canada). At least three pairs of animals were stained for each antibody and multiple sections were used for each trial.

4.3.7 OARSI scoring for DMM

For the DMM model, two blinded observers assigned a numerical grade and stage to each animal according to the Osteoarthritis Research Society International (OARSI) standards for OA histopathology (36). This was the same scoring system used in our previous rat studies (4, 5). Multiple sections were scored for each animal. One modification was made to the scoring system: a grade of 1 was assigned if the mouse cartilage had depleted safranin-O staining (representing depleted proteoglycan content) as this was determined to represent significant pathology, even in the absence of cartilage discontinuity. Scores were averaged for each group and statistical analysis was performed using a two-way ANOVA and Bonferroni post-tests.

4.3.8 OARSI scoring for spontaneous OA

One blinded observer assigned a semi-quantitative score to each animal based on the OARSI recommendations for histopathological assessment of OA in the mouse (17). Again, multiple sections were used for each animal. Since this scoring system is a variation of the one used to assess OA in the DMM model, no comparisons were made between the DMM animals and the eighteen month old mice. Scores were averaged for each group and statistical analysis was performed using a paired t-test.

4.4 Results

4.4.1 Young adult *Tgfa* null mice experience delayed OA progression in a DMM model

To examine the effects of *Tgfa* deficiency on post-traumatic OA in young adult mice, male 10 week-old heterozygote or homozygote mutant mice underwent DMM or sham surgery as depicted in Figure 4.1. Many disease features were apparent at the 7 week post-surgery time point in the DMM groups including chondrocyte clustering, hypertrophy, cell death, focal proteoglycan expression, and superficial zone fibrillation (Figure 4.2A). This indicated that DMM induced cartilage degeneration resembling OA pathology, as expected. The heterozygous DMM animals displayed more severe disease features including superficial zone delamination and lesions extending into the midzone (Figure 4.2A). All sham animals retained relatively healthy cartilage with scores below 6.0 (Figure 4.2A,B). At seven weeks post-surgery, the average OARSI score for heterozygous DMM mice was 13.7, while the score in the sham surgery group was 5.6 (Figure 4.2B). *Tgfa* knockout mice that received the DMM surgery showed significantly lower OARSI scores (average of 6.4) than their heterozygous littermates but had similar scores to sham-operated heterozygote and homozygote mice (Figure 4.2B).

We also examined molecular markers by immunohistochemistry to further validate our histological findings. Representative images show higher MMP13 staining in the articular cartilage of heterozygous DMM animals than in knockout DMM animals or in sham animals of either genotype (Figure 4.3A). Similarly, there appeared to be stronger type II collagen neoepitope staining in the cartilage of heterozygous DMM animals than in that of their knockout littermates or sham controls (Figure 4.3B).

Figure 4.1 A schematic representation of the post-traumatic and spontaneous aging models of OA used in this study. Ten-week old male *Tgfa* null and heterozygous mice underwent destabilization of medial meniscus (DMM) surgery. Two post-surgical time points were assessed with OARSI scoring: seven and fourteen weeks. Six-month-old *Tgfa* null and heterozygous mice also underwent DMM surgery and were assessed seven weeks post-surgery. Lastly, *Tgfa* null and heterozygous mice were housed for eighteen months with no surgical intervention, after which time spontaneous disease progression was assessed with OARSI scoring.

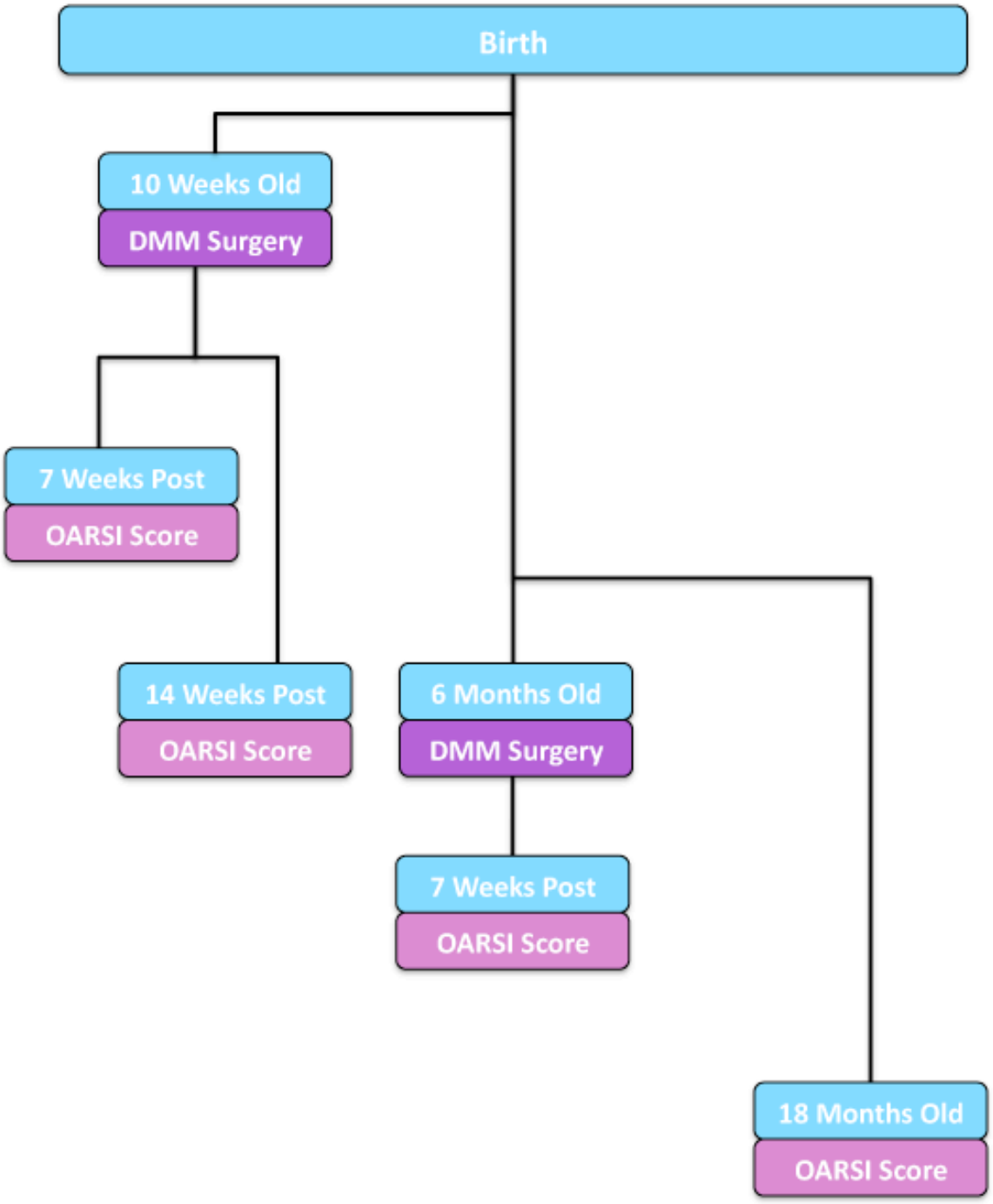


Figure 4.1 A schematic representation of the post-traumatic and spontaneous aging models of OA used in this study.

Figure 4.2 *Tgfa* knockout mice have lower OARSI scores than control littermates in a surgical model of OA. Ten-week-old *Tgfa* knockout mice and their heterozygous littermates received either surgery resulting in the destabilization of the medial meniscus (DMM) or sham surgery. At seven weeks post surgery, mice were sacrificed and their knee joints were prepared for histology. Tissues were stained with safranin-O and fast green (A) and then scored using the OARSI system for OA histopathology (B). Average OARSI scores +SEM are shown and indicate that *Tgfa* knockout mice have lower scores than OA heterozygous mice after DMM surgery (DMM groups n=5, sham groups n=4, *$p<0.05$).

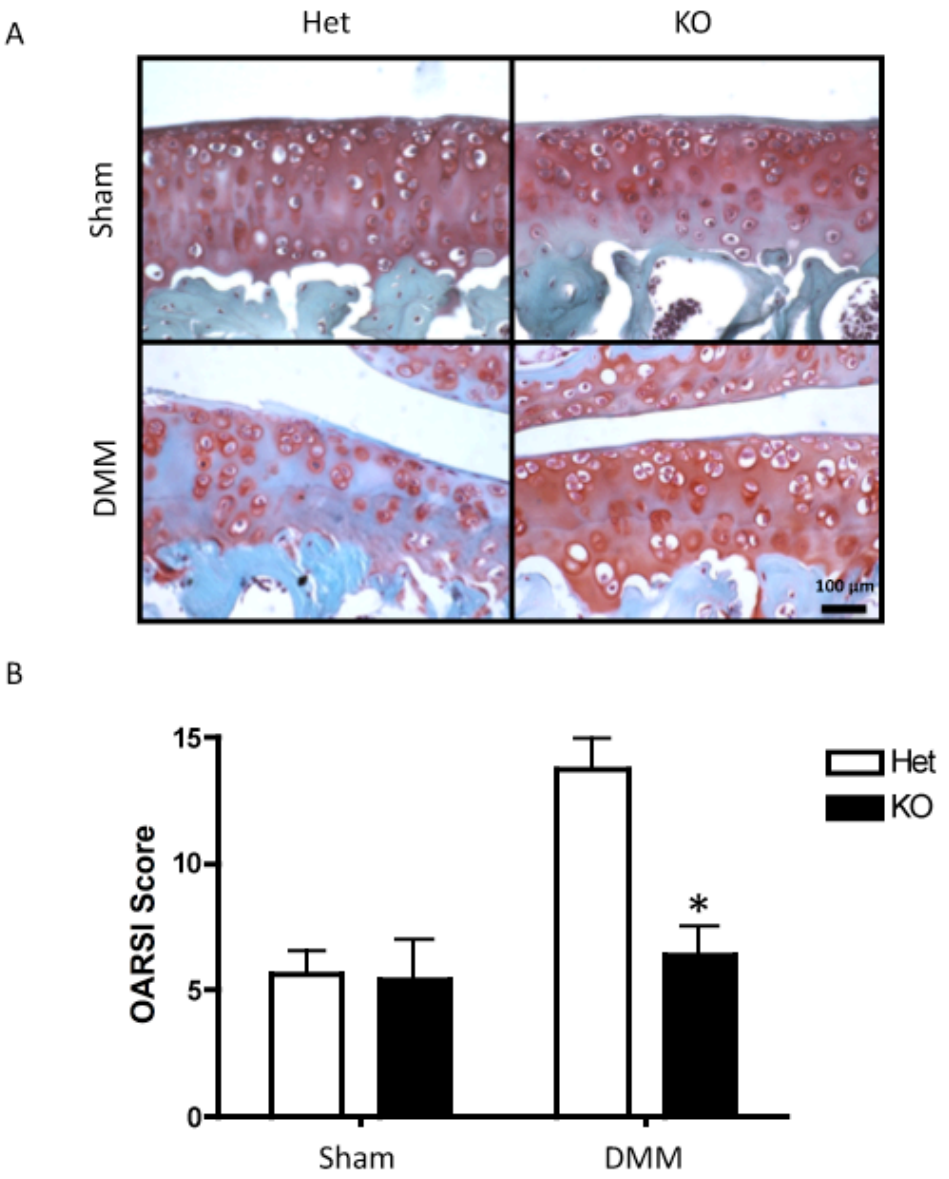


Figure 4.2 *Tgfa* knockout mice have lower OARSI scores than control littermates in a surgical model of OA.

Figure 4.3 *Tgfa* knockout mice express less MMP13 and less type II collagen neoepitopes than control littermates at 7 weeks post-surgery. Histological sections were prepared from the knee joints of both *Tgfa* knockout and heterozygous mice at seven weeks post-surgery. Immunohistochemistry was performed with primary antibodies against MMP13 and type II collagen fragments. This was followed by secondary antibody incubation and visualization with the substrate DAB (brown precipitate). Nuclei were counterstained with hematoxylin (blue). Representative images show that knockout animals express less MMP13 and type II collagen fragments than heterozygous controls.

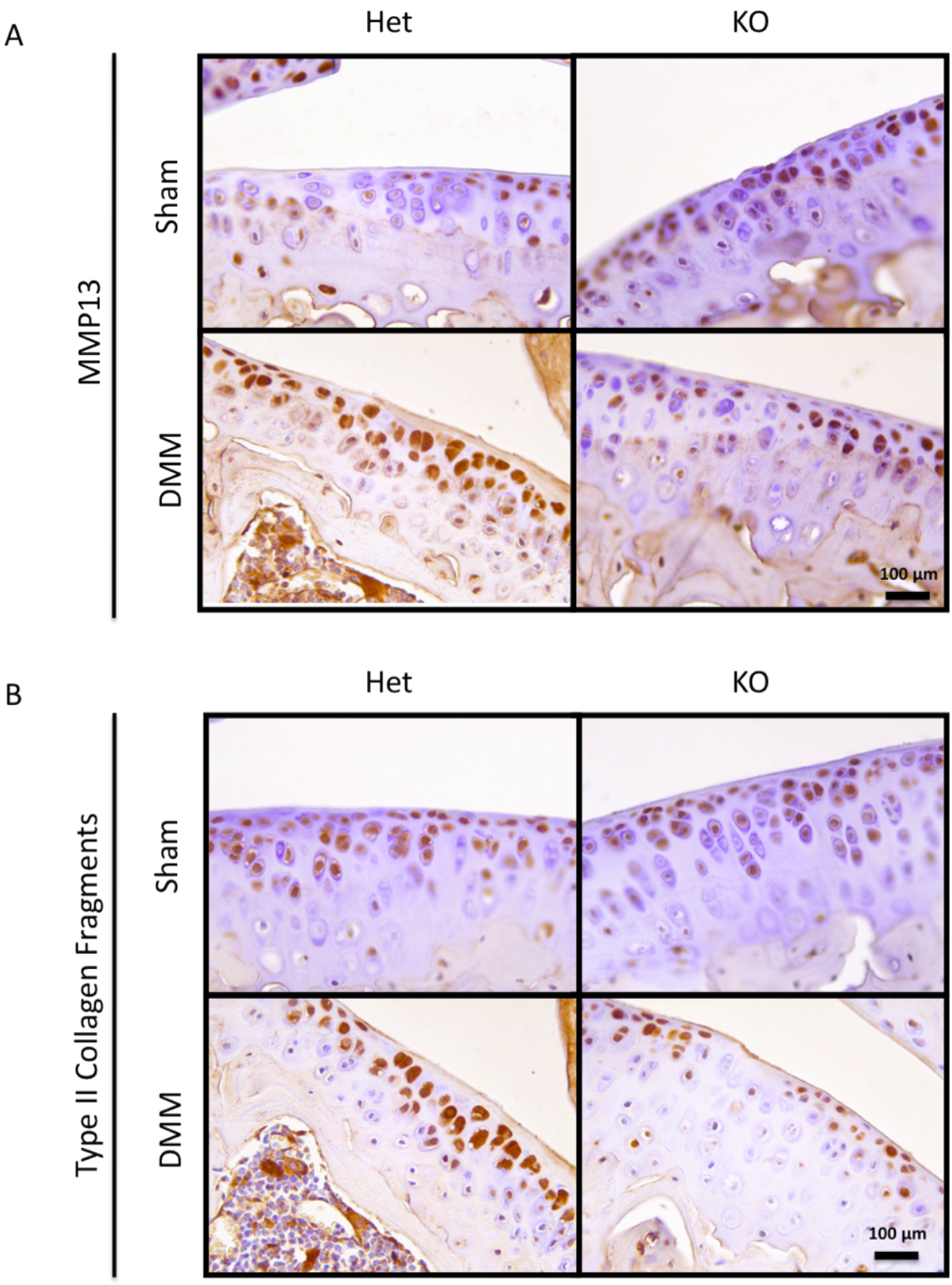


Figure 4.3 *Tgfa* knockout mice express less MMP13 and less type II collagen neoepitopes than control littermates at 7 weeks post surgery.

Similar results were seen when mice were analyzed fourteen weeks after surgery; heterozygous DMM animals had significantly higher scores (17.3) than knockout DMM animals (10.2) (Figure 4.4B). Many more DMM animals displayed advanced disease features such as superficial zone delamination and lesions extending into the midzone (Figure 4.4A), and some heterozygous animals displayed lesions extending to the calcified cartilage. Scores for both DMM groups were higher than at the seven week time point, indicating that the disease had progressed, while both sham groups had scores similar to those of the earlier time point (Figure 4.4B).

4.4.2 6-month-old *Tgfa* null mice are not protected from OA progression in a DMM model

These results suggested that loss of TGFα signaling protects from post-traumatic OA in young adults, a finding of significant impact for the human population because of the high number of sports-related injuries in this age group. However, humans joint injuries can also occur at later ages, thus combining two OA risk factors (aging and injury). To address this point, we performed DMM and sham surgery on six-month-old mice. OA progression was more advanced than when surgery was performed on younger mice (Figure 4.5A). Both *Tgfa* knockout mice and their heterozygous littermates had average OARSI scores of 17.1 when assessed at seven weeks post-surgery (Figure 4.5B). A number of disease features were evident including proteoglycan loss, superficial zone delamination and erosion of midzone cartilage (Figure 4.5A). In addition to the disease features mentioned above, some six moth old tissues revealed denudation to the calcified zone. *Tgfa* null mice were no longer protected from developing OA, since the knockout mice had scores and histopathological features similar to those of the heterozygous mice (Figure 4.5B).

Figure 4.4 *Tgfa* knockout mice still show resistance to developing OA at 14 weeks post-surgery. 10 week-old *Tgfa* knockout mice and their heterozygous littermates received DMM surgery or sham surgery. At fourteen weeks post surgery, mice were sacrificed and their knee joints were prepared for histology. Tissues were stained with safranin-O and fast green (A) and then scored using the OARSI system for OA histopathology (B). DMM knockout animals still displayed lower OARSI scores than their heterozygous control littermates at this time point (DMM groups n=6, sham groups n=4, *$p<0.05$).

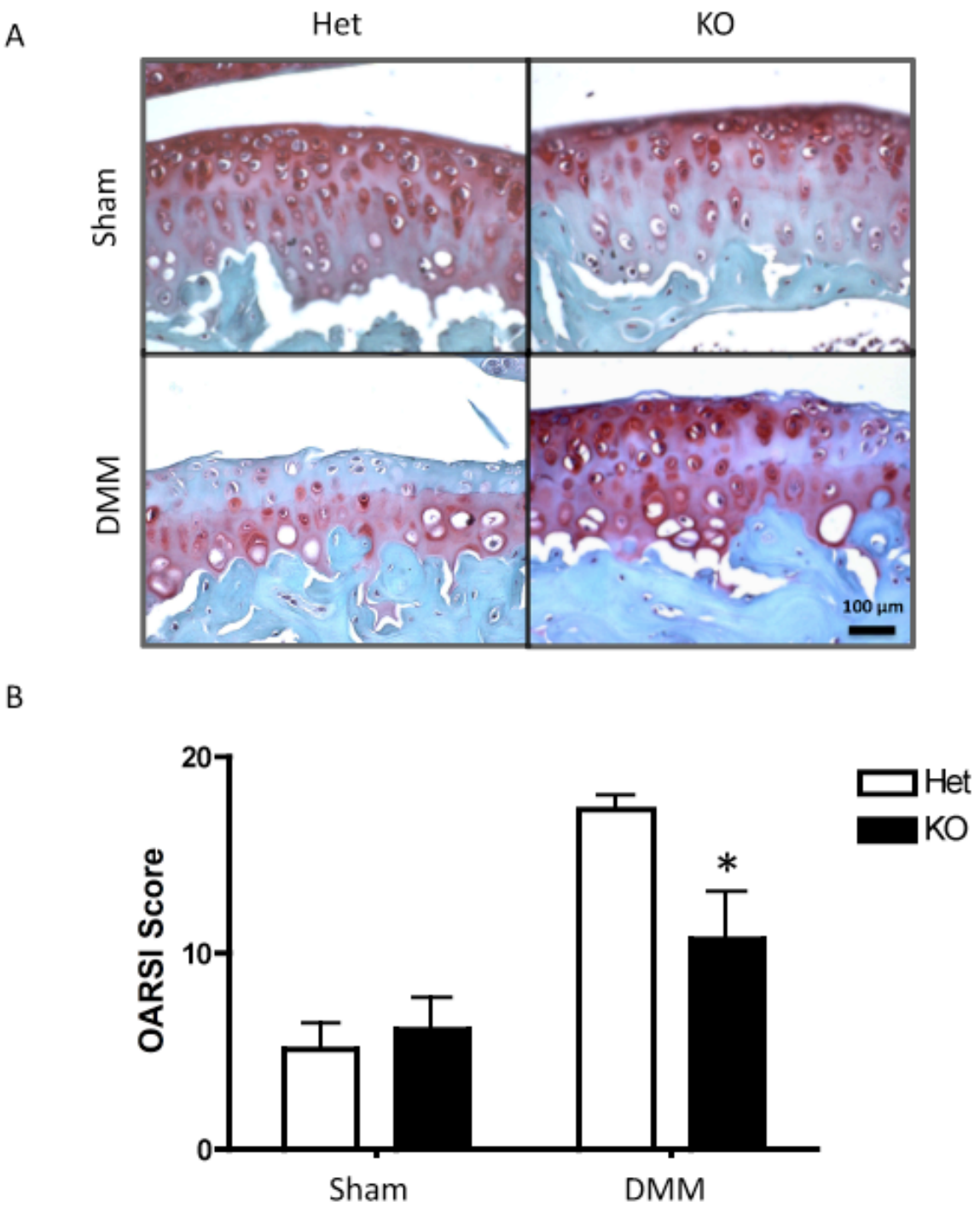


Figure 4.4 *Tgfa* knockout mice still show resistance to developing OA at 14 weeks post surgery.

Figure 4.5 There is no difference in disease progression between knockout and control animals after DMM surgery in middle-aged mice. Six-month-old *Tgfa* knockout and heterozygous mice received DMM or sham surgery. At seven weeks post surgery, these mice were sacrificed and their knee joints were isolated and prepared for OARSI scoring. Average OARSI scores +SEM are shown and indicate that DMM knockout and heterozygous mice are equally susceptible to developing OA in this model (sham groups n=4, DMM Het n=6, DMM KO n= 8, *$p<0.05$).

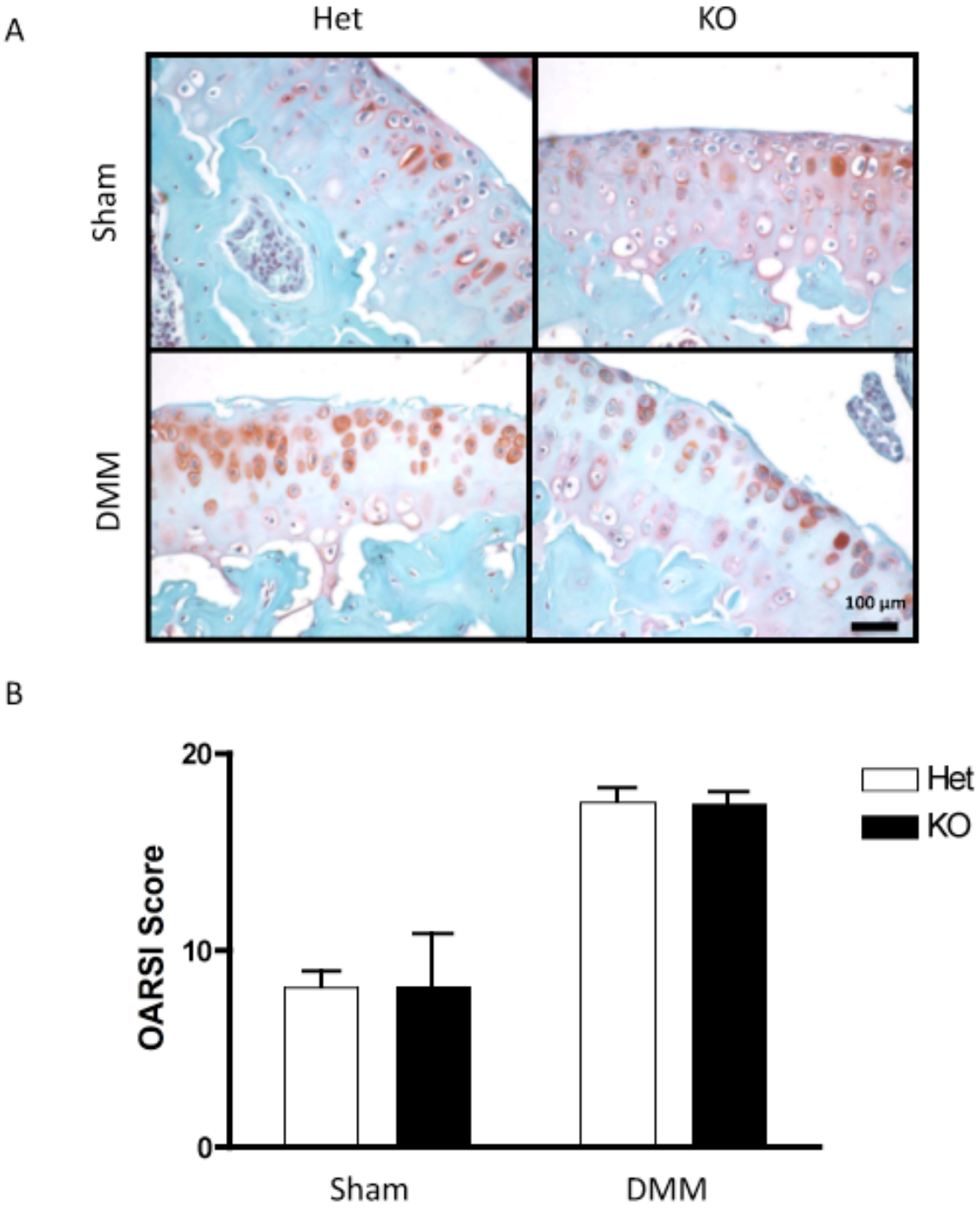


Figure 4.5 There is no difference in disease progression between knockout and control animals after DMM surgery in middle-aged mice.

4.4.3 *Tgfa* null mice are not protected from developing spontaneous OA

To address a potential role of TGFα in aging-associated OA, the knee, elbow, and ankle joints of eighteen-month-old mice were examined for spontaneous OA. Female mice were essentially completely protected from developing OA (data not shown). This finding has been well established in the literature and is attributed to difference in sex hormones (29). The knee joints of male mice showed the most advanced disease of the joints examined, revealing areas of proteoglycan loss, some areas of superficial zone delamination, and some loss of the midzone cartilage. Regardless of the genotype, however, articular cartilage remained largely intact, and areas of cartilage loss were focal in nature (Figure 4.6A). The elbow and the ankle cartilage for both *Tgfa* null and heterozygous mice was relatively healthy and showed only initial signs of OA, particularly proteoglycan loss (Figure 4.6B,C). Essentially all of the tissues examined from the elbow and the ankle had an intact articular cartilage surface, free of fibrillations and fissures (Figure 4.6B,C).

4.5 Discussion

Previously, our lab identified TGFα as a novel growth factor involved in the degeneration of articular cartilage (15, 16). In this study, we examined the role of TGFα in the progression of OA *in vivo* through the use of the *Tgfa* null mouse. We analyzed two separate models of OA: the post-traumatic DMM model as well as a spontaneous model of OA in aging mice. When young adult, ten-week old *Tgfa* null mice received DMM surgery, they were protected from developing OA at both post-surgical time points examined. While they still showed signs of degeneration, particularly at the later time

Figure 4.6 *Tgfa* knockout and heterozygous mice are equally susceptible to developing OA in an aging disease model. Male *Tgfa* knockout and heterozygous mice were housed until eighteen months of age. Animals were then sacrificed, and their knee (A), elbow (B) and ankle (C) joints were prepared for histology. Tissues were stained with safranin-O and fast green and scored using the OARSI recommendations for histopathological assessment of OA in the mouse. There were no differences in OARSI scores between genotypes for any of the joints examined (knee n=7, elbow n=4, ankle n=4, $p<0.05$).

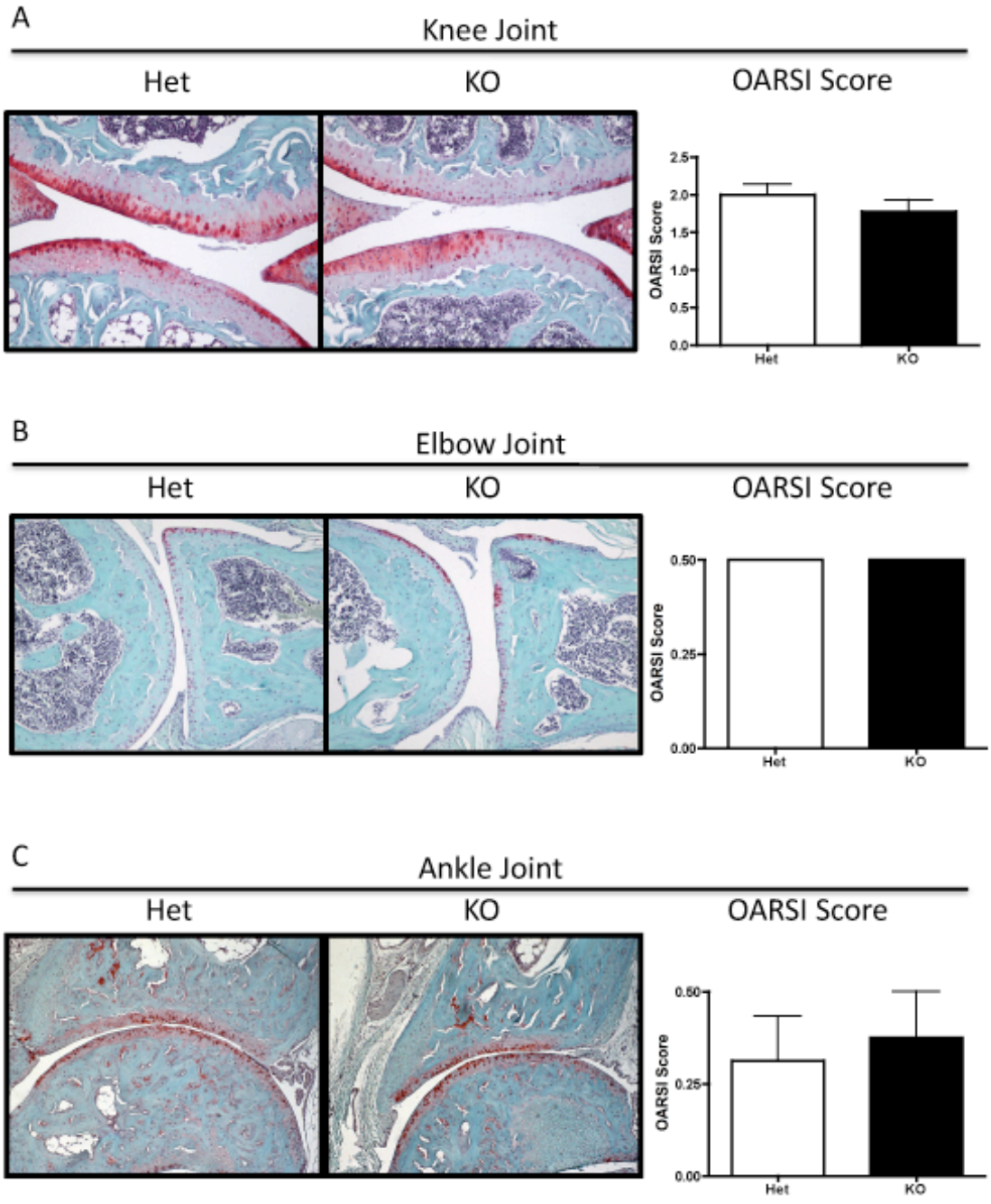


Figure 4.6 *Tgfa* knockout and heterozygous mice are equally susceptible to developing OA in an aging disease model.

point (14 weeks after surgery), disease progression was histologically less severe than that of control littermates. These data support our hypothesis, which is that in the absence of TGFα, mice are protected from OA development in a post-traumatic model. Our hypothesis was based on previous *in vitro* experiments that showed TGFα induced an OA-like phenotype in articular chondrocytes (7), as well as our findings of upregulation of *Tgfa* gene expression in a rat model of post-traumatic OA (4, 6). Specifically, TGFα caused a decrease in expression of Sox9, aggrecan, and type II collagen mRNA, and increased the expression of catabolic factors such as MMP13 (7). Thus by eliminating TGFα, we expected that we could reduce these overall catabolic events.

Contrary to our hypothesis however, the *Tgfa* null mice were not protected from developing OA in our spontaneous aging model or when DMM was performed in older mice. As mentioned previously, several changes occur in aging cartilage that could potentially make it more susceptible to injury, such as a more heavily cross-linked extracellular matrix, a loss in aggrecan content, and chondrocytes that are less responsive to anabolic factors (1). Furthermore, studies have shown that in the C56 BL/6 mouse articular cartilage thickness decreases with age, chondrocyte death increases, and the overall number of chondrocytes decreases as well (32). These changes within the tissue provide one potential explanation for why our older *Tgfa* null mice were more susceptible to or less protected from OA development. In addition to the articular cartilage itself, changes within the broader context of the joint are seen with aging. A recent study by Loeser et al. looked at the differences in gene expression within the entire joint in young and old mice receiving either sham or DMM surgery (28). In this particular study, twelve week old mice were used for the young group and twelve month old mice were used for

the aged group (28). Interestingly, many genes were found to be dysregulated between the young and aged groups post DMM surgery, as well as between the young and old sham-operated mice (28). This study clearly illustrates baseline differences in gene expression between joint tissues of young and old mice as well as differences in gene expression changes in response joint injury (28). Novel genes, including some related to muscle development and immune function, were identified thus highlighting the fact that tissues beyond the articular cartilage itself are likely important in the disease process (28). Since we did not specifically study other joint tissues (synovial membrane, subchondral bone, or surrounding muscles), it is possible that changes in these structures affected the overall function of the joint with age.

A study by Goekoop et al in 2005 examined a group of ninety-year-olds living without OA and identified several protective factors including male sex, a normal body mass index (BMI), and the absence of a family history of osteoarthritis (18). Obesity is a well-established risk factor for osteoarthritis, however its role in OA extends beyond mechanical effects, and a vast body of research is now focused on the link between fat metabolism and OA (15, 41). Furthermore, as people age, muscle mass generally declines and fat content increases (12). The animals in our studies were weighed at the time of surgery and sacrifice, however no differences were observed between the two genotypes (data not shown). While BMI and fat composition were not assessed quantitatively, older animals of both genotypes were observed to have more fat within their tissues upon dissection. Thus, perhaps the lack of protection seen in older *Tgfa* knockout mice could be attributed to changes relating to fat composition and metabolism.

There is additional evidence to suggest that TGFα and its downstream signaling pathway might be a good therapeutic target for OA therapy. TGFα is a member of the epidermal growth factor (EGF) family and signals through the EGF receptor (EGFR) (11, 23, 24). Recent studies have shown that mutant mice with enhanced EGFR signaling develop early, spontaneous degenerative disease in multiple joints the including knee, ankle, and temporomandibular joints (21, 50). These mice have a mutation in mitogen-inducible gene 6 (MIG6), also known as RALT or Gene 33 (50). MIG6 is a cytoplasmic protein that negatively regulates EGFR signaling through several mechanisms, including inhibition of the receptor kinase domains and receptor internalization and degradation (14, 19, 46, 48). These mutant mice have classical arthritic features such as loss of proteoglycan content, degradation of articular cartilage, formation of subchondral cysts, synovial hyperplasia, osteophyte formation, and abnormal calcification (21, 50). These studies suggest that increased EGFR signaling is sufficient to trigger spontaneous disease progression *in vivo* and consequently one might infer that blocking this signaling could prevent or delay disease progression. One difference between the MIG6 studies and our study is that in these models signaling from all EGFR ligands is inhibited while we specifically targeted TGFα. Thus, compensation by other EGF family members is another possible explanation for the lack of protection from OA in aging *Tgfa* null mice. As well, we have recently described a transient developmental bone phenotype in our *Tgfa* null mice that appears to resolve by ten weeks of age (the age that DMM surgeries are performed in this study) (42). However, we cannot be certain that the effects of this developmental phenotype, which include delayed osteoclast recruitment and decreased

MMP13 and RANKL (receptor activator of nuclear factor kappa B ligand) gene expression, do not have an effect on the outcome of our OA studies (42).

Our current results, however, suggest that targeting TGFα and its receptor might be beneficial in a subset of patients, potentially younger patients who have experienced a joint injury. It is possible that TGFα plays an important role post-trauma. We first identified TGFα as a growth factor involved in cartilage degeneration in microarray studies from another post-traumatic model of OA, namely the rat ACL transection/partial meniscectomy model (6). Our previous *in vitro* studies showed that in addition to the loss of chondrocyte phenotype, TGFα had an effect on chondrocyte proliferation both in primary articular chondrocyte cell culture and in an articular cartilage organ culture system (7). In the organ culture system, proliferation was evident based on the presence of chondroyte clusters, a well-established sign of early OA, believed to represent an attempt at cartilage repair (7, 40). Thus it is possible that TGFα is produced locally within the joint in response to injury, but lacks to ability to successfully repair cartilage. Our collaborators also examined TGFα mRNA in human cartilage collected at the time of joint replacement (7). Five of twelve arthritic samples expressed markedly increased TGFα mRNA levels, thus again suggesting that the growth factor plays an important role in a subset of patients (7).

Overall it appears that TGFα plays an important role in post-traumatic OA progression. Other factors, such as age however, also contribute to disease severity and progression. It may be important to take factors such as age and mechanism of joint injury into account when selecting patient for clinical trials for the development of DMOADs.

4.6 References

1. **Aigner T and Richter W.** OA in 2011: Age-related OA--a concept emerging from infancy? *Nat Rev Rheumatol* 8: 70-72, 2011.

2. **Aigner T, Sachse A, Gebhard PM, and Roach HI.** Osteoarthritis: pathobiology-targets and ways for therapeutic intervention. *Adv Drug Deliv Rev* 58: 128-149, 2006.

3. **Anderson DD, Chubinskaya S, Guilak F, Martin JA, Oegema TR, Olson SA, and Buckwalter JA.** Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention. *J Orthop Res* 29: 802-809, 2011.

4. **Appleton CT, McErlain DD, Henry JL, Holdsworth DW, and Beier F.** Molecular and histological analysis of a new rat model of experimental knee osteoarthritis. *Ann N Y Acad Sci* 1117: 165-174, 2007.

5. **Appleton CT, McErlain DD, Pitelka V, Schwartz N, Bernier SM, Henry JL, Holdsworth DW, and Beier F.** Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. *Arthritis Res Ther* 9: R13, 2007.

6. **Appleton CT, Pitelka V, Henry J, and Beier F.** Global analyses of gene expression in early experimental osteoarthritis. *Arthritis Rheum* 56: 1854-1868, 2007.

7. **Appleton CT, Usmani SE, Bernier SM, Aigner T, and Beier F.** Transforming growth factor alpha suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. *Arthritis Rheum* 56: 3693-3705, 2007.

8. **Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, and Poole AR.** Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99: 1534-1545, 1997.

9. **Bitton R.** The economic burden of osteoarthritis. *Am J Manag Care* 15: S230-235, 2009.

10. **Buckwalter JA and Martin JA.** Osteoarthritis. *Adv Drug Deliv Rev* 58: 150-167, 2006.

11. **Davis CG.** The many faces of epidermal growth factor repeats. *New Biol* 2: 410-419, 1990.

12. **Delmonico MJ, Harris TB, Visser M, Park SW, Conroy MB, Velasquez-Mieyer P, Boudreau R, Manini TM, Nevitt M, Newman AB, and Goodpaster BH.** Longitudinal study of muscle strength, quality, and adipose tissue infiltration. *Am J Clin Nutr* 90: 1579-1585, 2009.

13. **Englund M, Roemer FW, Hayashi D, Crema MD, and Guermazi A.** Meniscus pathology, osteoarthritis and the treatment controversy. *Nat Rev Rheumatol*, 2012.

14. **Fiorentino L, Pertica C, Fiorini M, Talora C, Crescenzi M, Castellani L, Alema S, Benedetti P, and Segatto O.** Inhibition of ErbB-2 mitogenic and transforming activity by RALT, a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. *Mol Cell Biol* 20: 7735-7750, 2000.

15. **Gkretsi V, Simopoulou T, and Tsezou A.** Lipid metabolism and osteoarthritis: lessons from atherosclerosis. *Prog Lipid Res* 50: 133-140, 2010.

16. **Glasson SS, Blanchet TJ, and Morris EA.** The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartilage* 15: 1061-1069, 2007.

17. **Glasson SS, Chambers MG, Van Den Berg WB, and Little CB.** The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage* 18 Suppl 3: S17-23, 2010.

18. **Goekoop RJ, Kloppenburg M, Kroon HM, Dirkse LE, Huizinga TW, Westendorp RG, and Gussekloo J.** Determinants of absence of osteoarthritis in old age. *Scand J Rheumatol* 40: 68-73, 2010.

19. **Hackel PO, Gishizky M, and Ullrich A.** Mig-6 is a negative regulator of the epidermal growth factor receptor signal. *Biol Chem* 382: 1649-1662, 2001.

20. **Hunter DJ.** Osteoarthritis. *Best Pract Res Clin Rheumatol* 25: 801-814, 2011.

21. **Jin N, Gilbert JL, Broaddus RR, Demayo FJ, and Jeong JW.** Generation of a Mig-6 conditional null allele. *Genesis* 45: 716-721, 2007.

22. **Krasnokutsky S, Samuels J, and Abramson SB.** Osteoarthritis in 2007. *Bull NYU Hosp Jt Dis* 65: 222-228, 2007.

23. **Kumar V, Bustin SA, and McKay IA.** Transforming growth factor alpha. *Cell Biol Int* 19: 373-388, 1995.

24. **Lee DC, Fenton SE, Berkowitz EA, and Hissong MA.** Transforming growth factor alpha: expression, regulation, and biological activities. *Pharmacol Rev* 47: 51-85, 1995.

25. **Leong DJ and Sun HB.** Events in articular chondrocytes with aging. *Curr Osteoporos Rep* 9: 196-201, 2011.

26. **Loeser RF.** Aging and osteoarthritis. *Curr Opin Rheumatol* 23: 492-496, 2011.

27. **Loeser RF.** Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators, and aging collide. *Arthritis Rheum* 54: 1357-1360, 2006.

28. **Loeser RF, Olex AL, McNulty MA, Carlson CS, Callahan MF, Ferguson CM, Chou J, Leng X, and Fetrow JS.** Microarray analysis reveals age-related differences in gene expression during the development of osteoarthritis in mice. *Arthritis Rheum* 64: 705-717, 2011.

29. **Ma HL, Blanchet TJ, Peluso D, Hopkins B, Morris EA, and Glasson SS.** Osteoarthritis severity is sex dependent in a surgical mouse model. *Osteoarthritis Cartilage* 15: 695-700, 2007.

30. **Makris EA, Hadidi P, and Athanasiou KA.** The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration. *Biomaterials* 32: 7411-7431, 2011.

31. **Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, and Dunn AR.** Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 73: 249-261, 1993.

32. **McNulty MA, Loeser RF, Davey C, Callahan MF, Ferguson CM, and Carlson CS.** Histopathology of naturally occurring and surgically induced osteoarthritis in mice. *Osteoarthritis Cartilage*, 2012.

33. **Mueller MB and Tuan RS.** Anabolic/Catabolic balance in pathogenesis of osteoarthritis: identifying molecular targets. *PM R* 3: S3-11, 2011.

34. **Muthuri SG, McWilliams DF, Doherty M, and Zhang W.** History of knee injuries and knee osteoarthritis: a meta-analysis of observational studies. *Osteoarthritis Cartilage* 19: 1286-1293, 2011.

35. **Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, and Laverty S.** Composition and structure of articular cartilage: a template for tissue repair. *Clin Orthop Relat Res*: S26-33, 2001.

36. **Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, Salter D, and van den Berg WB.** Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage* 14: 13-29, 2006.

37. **Qvist P, Bay-Jensen AC, Christiansen C, Dam EB, Pastoureau P, and Karsdal MA.** The disease modifying osteoarthritis drug (DMOAD): Is it in the horizon? *Pharmacol Res* 58: 1-7, 2008.

38. **Salaffi F, Carotti M, Stancati A, and Grassi W.** Health-related quality of life in older adults with symptomatic hip and knee osteoarthritis: a comparison with matched healthy controls. *Aging Clin Exp Res* 17: 255-263, 2005.

39. **Sandell LJ.** Etiology of osteoarthritis: genetics and synovial joint development. *Nat Rev Rheumatol* 8: 77-89, 2012.

40. **Sandell LJ and Aigner T.** Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 3: 107-113, 2001.

41. **Sowers MR and Karvonen-Gutierrez CA.** The evolving role of obesity in knee osteoarthritis. *Curr Opin Rheumatol* 22: 533-537, 2010.

42. **Usmani SE, Pest MA, Kim G, Ohora SN, Qin L, and Beier F.** Transforming growth factor alpha controls the transition from hypertrophic cartilage to bone during endochondral bone growth. *Bone* 51: 131-141, 2012.

43. **van der Waal JM, Terwee CB, van der Windt DA, Bouter LM, and Dekker J.** The impact of non-traumatic hip and knee disorders on health-related quality of life as measured with the SF-36 or SF-12. A systematic review. *Qual Life Res* 14: 1141-1155, 2005.

44. **Welch ID, Cowan MF, Beier F, and Underhill TM.** The retinoic acid binding protein CRABP2 is increased in murine models of degenerative joint disease. *Arthritis Res Ther* 11: R14, 2009.

45. **Woolf AD and Pfleger B.** Burden of major musculoskeletal conditions. *Bull World Health Organ* 81: 646-656, 2003.

46. **Xu D, Makkinje A, and Kyriakis JM.** Gene 33 is an endogenous inhibitor of epidermal growth factor (EGF) receptor signaling and mediates dexamethasone-induced suppression of EGF function. *J Biol Chem* 280: 2924-2933, 2005.

47. **Zhang W, Moskowitz RW, Nuki G, Abramson S, Altman RD, Arden N, Bierma-Zeinstra S, Brandt KD, Croft P, Doherty M, Dougados M, Hochberg M, Hunter DJ, Kwoh K, Lohmander LS, and Tugwell P.** OARSI recommendations for the management of hip and knee osteoarthritis, Part II: OARSI evidence-based, expert consensus guidelines. *Osteoarthritis Cartilage* 16: 137-162, 2008.

48. **Zhang X, Pickin KA, Bose R, Jura N, Cole PA, and Kuriyan J.** Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. *Nature* 450: 741-744, 2007.

49. **Zhang Y and Jordan JM.** Epidemiology of osteoarthritis. *Clin Geriatr Med* 26: 355-369, 2010.

50. **Zhang YW, Su Y, Lanning N, Swiatek PJ, Bronson RT, Sigler R, Martin RW, and Vande Woude GF.** Targeted disruption of Mig-6 in the mouse genome leads to early onset degenerative joint disease. *Proc Natl Acad Sci U S A* 102: 11740-11745, 2005.

Chapter 5

5 Discussion

5.1 Overview

The overall objective of my thesis was to gain a better understanding of the role of transforming growth factor alpha (TGFα) in cartilage biology, both in the cartilage growth plate during endochondral bone development and in degenerating articular cartilage during the disease state of osteoarthritis (OA). My laboratory first became interested in TGFα after my colleagues performed microarray studies on articular cartilage isolated from a surgical rat model of OA (6, 7). TGFα mRNA levels were elevated almost 4-fold in the OA animals compared controls (6). The laboratory decided to pursue this growth factor further since its role in normal cartilage biology was not fully understood and since it had not previously been implicated in OA initiation or progression. Initial *in vitro* investigations showed that TGFα had a profound effect on the chondrocyte phenotype: TGFα treatment caused changes in chondrocyte morphology, induced proliferation, and shifted synthesis away from anabolic factor expression towards that of catabolic factor expression (8). The next logical step was to investigate possible downstream pathways that might regulate TGFα-induced effects on the chondrocyte phenotype.

The first study in my thesis investigates the link between TGFα and the endothelin receptor A (ET(A)R) signaling system. Just like TGFα, the gene for ET(A)R denoted as *Ednra*, was upregulated in the original rat model of OA (6). There is also a body of literature implicating ET(A)R in both OA and normal chondrocyte aging (30-34, 40, 45, 46, 51). Since age is one of the most common risk factors for OA, I decided that this

would be a good downstream candidate target to focus on. Furthermore, ET(A)R is known to interact with the receptor through which TGFα signals, the epidermal growth factor receptor (EGFR), in other diseases such as cancer (10, 37). I subsequently performed *in vitro* experiments with primary chondrocyte cell cultures and osteochondral organ culture explants. I found that TGFα induced ET(A)R expression at both the mRNA and protein level. However, inhibiting ET(A)R with a non-specific ET(A/B)R antagonist only partially blocked the TGFα effects in our explant model. Thus while ET(A)R appears to be a downstream target of TGFα, the signaling pathways affected by this growth factor are clearly more complex.

Next I wanted to examine TGFα function in the skeleton using an *in vivo* system. Therefore the third and fourth chapters of this thesis involve the *Tgfa* knockout mouse previously characterized by Mann et al. (41). The major phenotype reported in this mouse is the presence of a wavy coat due to the misalignment of hair follicles (41). No mention of bone or cartilage abnormalities is made. Previous *in vitro* literature, on the other hand, does suggest a role for TGFα in bone development and turnover. Studies indicate that TGFα has a negative effect on chondrogenesis of limb bud mesenchymal cells (14, 23, 64). As well, research has shown that TGFα stimulates bone resorption and inhibits bone formation *in vitro* (25, 26). The role of TGFα during endochondral bone development has not been explicitly examined however, and this led me to my second major study.

I examined the cartilage growth plates of *Tgfa* wildtype, heterozygous, and null mice over a variety of time points ranging from newborn to ten weeks of age. Interestingly, *Tgfa* null mice were found to have a transient growth plate phenotype characterized by an

enlarged hypertrophic zone and decreased numbers of osteoclasts at the cartilage/bone interface. Analysis of gene expression revealed that these mice also express less matrix metalloproteinase 13 (MMP13) as well as less receptor activator of nuclear factor kappa-B ligand (RANKL) mRNA than their heterozygous and wild type littermates. RANKL is an important activator of osteoclastogenesis and chondrocyte-produced RANKL has been shown to stimulate osteoclast formation (35, 57). MMP13 also plays an important role in extracellular matrix breakdown during endochondral bone formation, and is required for normal vascularization and subsequent bone remodeling (27, 56). Thus I demonstrated a previously unrecognized growth plate phenotype in the *Tgfa* knockout mouse and reported TGFα's regulatory role in the transition from cartilage to bone.

Lastly, I wanted to examine the role of TGFα in the disease state of osteoarthritis *in vivo*. I used the well-established destabilization of medial meniscus model (DMM) to represent a slowly progressing model of post-traumatic OA (19). Furthermore, I examined spontaneous disease progression in aging animals. Due to the overall catabolic effect of TGFα in articular chondrocytes and cartilage explants *in vitro*, I hypothesized that *Tgfa* null mice would be protected from OA progression in both the injury and aging models. I found that young knockout mice were protected in the DMM model, however aging knockout mice developing spontaneous disease as well as older knockout mice receiving DMM surgery were not protected from OA progression. This study demonstrates that TGFα may be an important therapeutic target in post-traumatic forms of OA, however its role may not be as significant when other risk factors such as age come into play. This study also reminds us that OA is a complex multifactorial disease and that treatments may need to be specific to the main etiological risk factors involved.

Overall, these data demonstrate an important role for TGFα both in the conversion of cartilage to bone during endochondral ossification, and in cartilage degeneration in osteoarthritis. A common thread between development and disease, is that both of these mechanisms appear to be driven in part through the production of catabolic factors such as MMP13.

5.2 Contributions and Significance of Findings

5.2.1 Contributions to the Field of Skeletal Biology

In this thesis, I characterize for the first time the growth plate phenotype of TGFα null mice. Prior to these studies, the role of TGFα in the cartilage growth plate had not been explicitly investigated. As mentioned above however, previous work did highlight TGFα's negative effect on chondrogenesis and its stimulatory effect on osteoclastogenesis (14, 23, 25, 26, 64). I describe the transient growth plate phenotype of *Tgfa* null mice that includes shorter tibiae and humeri, an enlarged hypertrophic zone and overall growth plate length, delayed primary and secondary ossification, and reduced osteoclast staining at the cartilage/bone junction. To complement the decrease in osteoclasts, I also demonstrate a decrease in *Mmp13* and *Rankl* gene expression. The phenotype seen is very similar to that of the *Efgr* knockout mouse (61). Since these mice do not survive long after birth, they were consequently studied during embryonic development only. Their growth plate phenotype, however, is very similar to that seen in my studies and is characterized by delayed primary ossification, an enlarged hypertrophic zone, and decreased osteoclast recruitment (61). This study examined the inactivation of the EGF receptor and thus the identity of the ligand or ligands involved remained unknown. The bone phenotypes of other EGFR ligand knockout mice have been

characterized, including amphiregulin (AREG) and betacellulin (BTC) (52). Neither of these models however, has reported a growth plate phenotype as seen in the *Egfr* knockout mouse. Thus it is likely that TGFα is one of the main ligands responsible for the roles of EGFR during endochondral ossification. In addition, my studies provide mechanistic insights into the observed phenotype, namely through the reduced expression of MMP13 and RANKL in cartilage of *Tgfa* null mice.

My collaborators recently published a study exploring the inactivation of EGFR at post-natal time points in the rat through the use of an EGFR specific inhibitor, gefitinib (66). Their study complements the *Tgfa* knockout model in that the gefitinib-treated rats developed an expanded cartilage growth plate with an accumulation of hypertrophic cells, and had decreased osteoclasts at the osteochondral junction (66). The rats also expressed less *Rankl* and *Mmp13* than untreated animals (66). To determine whether these effects were due to primary effects on the growth plate, my collaborators also created cartilage-specific *Egfr* knockout mice using the *Col2a1*-promoter to drive Cre recombinase expression (66). Again, similar changes in the growth plate were observed, thus revealing that EGFR inactivation directly affects the cartilage growth plate. The overlap observed between the TGFα knockout mouse and models of EGFR inactivation further suggests that TGFα is the main mediator of EGFR effects in the cartilage growth plate.

In addition to these findings, I implicate TGFα in the development of the secondary ossification centre for the first time. *Tgfa* null mice show delay in secondary ossification when compared to their control littermates. When I performed immunohistochemistry (IHC) in the secondary ossification centre with an antibody specific for the blood vessel

marker platelet endothelial cell adhesion molecule 1 (PECAM-1), I saw reduced levels of staining in the mutant animals. Thus in addition to its role in the primary ossification centre, I have demonstrated a role for TGFα in secondary ossification development.

Lastly, through *in vivo* embryonic tibia organ culture studies, I show that TGFα affects genes related to chondrocyte hypertrophy. Specifically, TGFα decreases expression of early hypertrophic markers including *p57* and *ColX*, and increases the expression of late hypertrophic markers including the *Mmp9*, *Mmp13*, and *Mmp14*. These results suggest a novel role for TGFα in promoting the transition from early hypertrophic to terminally differentiated chondrocytes. Collectively these data contribute to the growing understanding of molecular mechanisms controlling the organization and regulation of the cartilage growth plate. They also give us insight into how TGFα might contribute to cartilage pathology in osteoarthritis, namely through catabolic factor expression and potentially through other mechanisms such as chondrocyte hypertrophy.

5.2.2 Contributions to Field of Osteoarthritis

I demonstrate that ET(A)R is a downstream target of TGFα. Furthermore I demonstrate this in two different species (rat and mouse) and in tissues of varying ages (embryonic, newborn, and four to five month old tissues). My *in vitro* experiments show that TGFα upregulates ET(A)R in articular chondrocytes at both the mRNA and protein level. This is consistent with previous work, demonstrating that a number of growth factors and cytokines including EGF are able to induce ET(A)R expression in articular chondrocytes (46). Inhibiting endothelin signaling with a non-specific ET(A/B)R inhibitor however, only partially blocks TGFα catabolic effects in articular cartilage, namely the expression

of MMP13 and subsequent breakdown of type II collagen. Thus there must be other signaling pathways downstream of TGFα capable of mediating its OA-like phenotype. Additional work done in my lab demonstrates that various pathways are responsible for mediating TGFα effects in articular chondrocytes. For example, RhoA/ROCK has been shown to mediate TGFα -induced morphologic changes in chondrocytes, in agreement with previous studies demonstrating important roles of this pathway in chondrogenesis (60, 62). The MEK/ERK pathway mediates the downregulation of anabolic gene expression by TGFα, again fitting with known roles of this pathway in chondrocytes (11, 55). Both the Rho/ROCK and MEK/ ERK pathway also regulate type II collagen cleavage and aggrecan breakdown in articular cartilage (9). Thus while TGFα is capable of inducing ET(A)R expression in articular chondrocytes, this is only one signaling pathway of many involved in the induction of an OA-like phenotype.

In addition, I also show an increase in inducible nitric oxide synthase (iNOS) staining in IHCs from the TGFα- treated organ culture studies. To my knowledge, this is the first time that TGFα has been shown to effect parameters of nitric oxide (NO) metabolism in articular chondrocytes. This is significant as the increase in iNOS may contribute to NO production and potentially serve as another mechanism through which TGFα carries out its catabolic effects (5, 47, 58). Recently, a study by Kaufman et al complemented my ET(A)R studies nicely (29). This group demonstrated that inhibition of the ET(A)R with a specific inhibitor (BQ-123) in a surgical model of OA prevented disease progression. A similar model of OA was used (rat ACL transection) as in my laboratory's original studies (6, 7).

My *in vivo* models of OA also provide novel information to the field. I demonstrate that young adult *Tgfa* null mice are protected from developing OA in a DMM model of the disease, both at seven and fourteen weeks post-surgery. Through this *in vivo* model, I have validated TGFα as a potential therapeutic target for the development of a disease modifying osteoarthritic drug (DMOAD). Experts in the field support the use of *in vivo* surgical disease models to validate potential therapeutic OA targets (17). For example, DMM surgery was performed in the ADAMTS-5 (a disintegrin and metalloproteinase with thrombospondin motifs) knockout mouse, and observations of delayed disease progression now make it one of the most attractive targets for DMOAD development (18, 48).

Prior to the microarray studies, TGFα had not been implicated in the disease process. Previous research did however, report the presence of TGFα in the synovial fluid and synovial membranes of arthritic patients with higher levels observed in individuals with rheumatoid arthritis (RA) than in those with OA (22, 36). TGFα has also been detected in control synovium and synovial fluid, suggesting a normal physiological role within the joint (22). Evidence does exist however, for the involvement of EGFR in OA development. Recent studies involving mice with mutations in mitogen-inducible gene 6 (MIG6) have reported spontaneous degenerative joint disease in these animals (67). MIG6 (also known as RALT or Gene 33) is a cytoplasmic protein that negatively regulates EGFR signaling by several mechanisms (16, 21, 63, 65). Homozygous null mutants of the *Mig6* allele develop early degenerative disease in the knee, ankle, and temporomandibular joints (28, 67). As well, the *Mig6* null joints show classical arthritic features including depletion of proteoglycans, degradation of articular cartilage,

formation of subchondral cysts, synovial hyperplasia, osteophyte formation, and abnormal calcification (28, 67). These studies illustrate that enhanced EGFR signaling is sufficient to trigger spontaneous disease progression *in vivo* and thus complement my knockout studies, in which decreased EGFR ligand is protective.

My studies also reveal that the protection from OA is not universal in all osteoarthritic models. In the aging models (spontaneous OA development and DMM surgery performed in older mice) the *Tgfa* knockout mice are no longer protected from disease progression, and they develop OA that is histologically indistinguishable from control mice. Age is a major risk factor for the development of OA, and specifically with meniscal injuries, it has been shown that the tempo of disease progression varies with age (44, 50). Patients who sustain a meniscal injury between the ages of seventeen and thirty develop radiologic signs of OA on average after fifteen years, while patients who sustain the same injury over the age of thirty develop signs of OA only five years later (50). Our data fit with this clinical picture as the aging mice show more aggressive lesions post-surgery, and all aging mice regardless of genotype are susceptible to disease progression. These data also contribute to the theory that OA is a heterogeneous disease based on specific etiological risk factors (i.e. obesity, aging, injury) and that DMOADs should be developed with these sub-phenotypes of OA in mind (12). In the case of TGFα, the evidence thus far suggests that it might be an important therapeutic target in post-traumatic cases of OA in young individuals.

5.3 Limitations of Research

5.3.1 Limitations of In Vitro Models

I acknowledge that these studies have several limiting factors. Firstly, my *in vitro* experiments are clearly limited by their ability to recapitulate the *in vivo* environment. For the ET(A)R studies I used both primary chondrocyte cultures and osteochondral explant cultures. The primary chondrocyte cultures were isolated from the distal femoral condyles of neonatal rats and plated in monolayer culture as previously described (53). The benefit of this culture system is that a large number of cells can be isolated at once; chondrocyte isolation would be far more challenging from mature articular cartilage with sparse cellular density. However, the major drawback is that these cells are very young, and as previous stated, differences in gene expression, responses to growth factors, and receptor profiles are seen with aging chondrocytes (38, 45, 46). This culture system may not have been the best choice for my ET(A)R studies as the receptor density is known to increase significantly with aging chondrocytes (45, 46). Furthermore, monolayer cultures lack interaction with an extracellular matrix. Consequently, cell surface receptors such as integrins that would normally interact with the matrix, likely have altered signaling in this context (20).

The osteochondral explants address some of these limitations (i.e. they are derived from adult animals and retain an intact extracellular matrix), but they still lack the influence of mechanical forces. Chondrocytes are known to response to various forces through integrin receptors, stretch-activated calcium ion channels, and deformation of the chondrocyte cytoskeleton (2, 4, 13, 20). Lastly, these *in vitro* models lacked the context of the joint as a unit. It is well established that OA is a disease affecting the entire joint

including the articular cartilage, synovial membrane, joint capsule, and subchondral bone (3, 24). By examining effects on articular chondrocytes or cartilage alone we lose potentially important cross-talk between these tissues. Future *in vivo* studies using pharmacological or genetic manipulation of the ET(A)R signaling system would complement our studies, but as outlined, some of these have been performed by others recently (29).

The non-specific ET(A/B)R inhibitor Bosentan that was used in my studies also provides some concerns. Bosentan is a dual endothelin receptor A/B antagonist which is clinically used for the treatment of pulmonary arterial hypertension (43). We chose to use this drug since it has already been approved for use clinically. However, the antagonist is not selective for ET(A)R and thus it is possible that some of the effects we saw in chondrocyte cell culture and organ culture were due to inactivation of ET(B)R and its downstream pathways. Use of receptor-specific antagonists such as BQ-123 would be one potential future application.

5.3.2 Limitations of In Vivo Models

In my growth plate studies, RNA was isolated from newborn (P0) mice since cartilage is expansive and easily accessible at this stage. It is difficult to distinguish the growth plate cartilage from what will become the articular cartilage at this stage since the secondary ossification centre (which divides these two regions) has not yet developed. While the mutant phenotype is predominantly present in the hypertrophic zone of the growth plate, I decided to take all cartilage from P0 tibia since it is plausible that factors regulating this phenotype may come from other areas of the growth plate or other chondrocytes in general. It is possible that some areas of the growth plate might have been missed during

isolation, especially some of the cartilage adjacent to the mineralized zone. Furthermore, it is possible that other tissues such as perichondrium were isolated along with the cartilage. I did my best to remove all excess tissues and collect the complete growth plate, however it is possible that some of the gene changes we observed were in part due to additional tissues.

I used full body *Tgfa* knockout mice in my experiments, in which the *Tgfa* allele was disrupted by the insertion of a neomycin cassette (41). Since I did not use a tissue-specific knockout model, I cannot be completely confident that the developmental phenotype I have characterized is due to direct effects of the absence TGFα of on the growth plate. Rather, it is possible that these effects are mediated by some other systemic factors influenced by the null mutation. My collaborators however, have created a cartilage-specific *Egfr* knockout mouse that has a similar phenotype to the *Tgfa* null mice, thus suggesting that my observations may indeed be due to direct effects (66). Furthermore, while I describe a transient developmental phenotype in these mice that resolves by ten weeks of age, I cannot be absolutely certain that this phenotype does not in some way persist or affect the OA studies. My conclusion that TGFα is a novel therapeutic DMOAD target, assumes that the developmental phenotype does not in some way affect OA susceptibility in our knockout animals. In other words, unlike a drug that could be administered to patients at the time of joint injury, our mice have always had their mutation, and this fact may affect the clinical relevance of our study.

My collaborators (Qin and colleagues, University of Pennsylvania) performed micro CT analysis on aging animals and found that the *Tgfa* null skeleton appears to lose both cortical area and trabecular volume over time (data not shown). I need to perform more

studies to correctly interpret these results, however it is evident that there are still some effects of the *Tgfa* mutation even in mice older than ten weeks of age.

The DMM surgery provides a mild model of post-traumatic OA (19). It is relevant as meniscal injuries are almost always seen with radiographic signs of OA, and as the meniscus is the most commonly injured intra-articular tissue (15, 39). I also employed an aging model of OA by analyzing spontaneous joint disease. While I limited my analysis to these two models, there are numerous additional risk factors that contribute to OA including obesity, female gender, and repetitive mechanical loading. From this thesis, my interpretations are limited to the role of TGFα in post-traumatic OA and aging as I did not take these other factors into account. Currently however, some of my colleagues are investigating the effects of a high fat diet on joint pathology in various genetically modified mice, including the *Tgfa* mutants.

5.4 Future Directions

Several possible future projects could be explored based on the results of this thesis. Firstly, to confirm both the *Tgfa* null growth plate phenotype and the results from our OA models, it would be useful to create a cartilage-specific *Tgfa* knockout mouse using the Cre-loxP system with Cre recombinase expressed under control of the *Col2a1* promoter, as our laboratory has done for other genes (54, 59). In addition, it would be beneficial to examine multiple joint tissues including the subchondral bone, synovium, and synovial fluid. By examining the entire joint, one may gain a better understanding of how these tissues interact and signal to each other throughout disease progression. Furthermore, I could add gait analysis to these studies to assess joint function. Thus far I have been

focusing on cartilage damage as the main endpoint. However structural changes alone are not sufficient for a clinical diagnosis of the OA.

It might also be worthwhile to create an inducible, tissue-specific ET(A)R knockout mouse. Full body ET(A)R knockout mice do not survive long after birth due to respiratory distress and they also display craniofacial abnormalities (1). While another group has already demonstrated that OA progression can be delayed in an ACL transection model of OA with ET(A)R inhibition, it might be of value to determine whether knocking out this receptor later in life prevents spontaneous disease progression. This would be particularly interesting since ET(A)R expression in chondrocytes appears to increase with age (45, 46).

Lastly, it would be very interesting to explore the role of TGFα in other forms of joint disease such as RA. As previously mentioned, TGFα has been identified in synovial fluid and synovial membranes of both OA and RA patients, and higher levels were actually observed in individuals with RA (22, 36). Furthermore, studies have shown that bone destruction in RA may be mediated by osteoclasts, and that chondrocyte-produced RANKL may contribute to the activation of cells and subsequent juxta-articular bone loss (42, 49). These findings suggest that TGFα could affect RA and possibly OA through effects on osteoclasts.

5.5 References

1. **Abe M, Ruest LB, and Clouthier DE.** Fate of cranial neural crest cells during craniofacial development in endothelin-A receptor-deficient mice. *Int J Dev Biol* 51: 97-105, 2007.

2. **Aigner T, Kurz B, Fukui N, and Sandell L.** Roles of chondrocytes in the pathogenesis of osteoarthritis. *Curr Opin Rheumatol* 14: 578-584, 2002.

3. **Aigner T, Sachse A, Gebhard PM, and Roach HI.** Osteoarthritis: pathobiology-targets and ways for therapeutic intervention. *Adv Drug Deliv Rev* 58: 128-149, 2006.

4. **Alenghat FJ and Ingber DE.** Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins. *Sci STKE* 2002: pe6, 2002.

5. **Amin AR and Abramson SB.** The role of nitric oxide in articular cartilage breakdown in osteoarthritis. *Curr Opin Rheumatol* 10: 263-268, 1998.

6. **Appleton CT, McErlain DD, Henry JL, Holdsworth DW, and Beier F.** Molecular and histological analysis of a new rat model of experimental knee osteoarthritis. *Ann N Y Acad Sci* 1117: 165-174, 2007.

7. **Appleton CT, Pitelka V, Henry J, and Beier F.** Global analyses of gene expression in early experimental osteoarthritis. *Arthritis Rheum* 56: 1854-1868, 2007.

8. **Appleton CT, Usmani SE, Bernier SM, Aigner T, and Beier F.** Transforming growth factor alpha suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. *Arthritis Rheum* 56: 3693-3705, 2007.

9. **Appleton CT, Usmani SE, Mort JS, and Beier F.** Rho/ROCK and MEK/ERK activation by transforming growth factor-alpha induces articular cartilage degradation. *Lab Invest* 90: 20-30, 2010.

10. **Bagnato A and Rosano L.** The endothelin axis in cancer. *Int J Biochem Cell Biol* 40: 1443-1451, 2008.

11. **Beier F and Loeser RF.** Biology and pathology of Rho GTPase, PI-3 kinase-Akt, and MAP kinase signaling pathways in chondrocytes. *J Cell Biochem* 110: 573-580, 2010.

12. **Bijlsma JW, Berenbaum F, and Lafeber FP.** Osteoarthritis: an update with relevance for clinical practice. *Lancet* 377: 2115-2126, 2011.

13. **D'Andrea P, Calabrese A, Capozzi I, Grandolfo M, Tonon R, and Vittur F.** Intercellular Ca2+ waves in mechanically stimulated articular chondrocytes. *Biorheology* 37: 75-83, 2000.

14. **Dealy CN, Scranton V, and Cheng HC.** Roles of transforming growth factor-alpha and epidermal growth factor in chick limb development. *Dev Biol* 202: 43-55, 1998.

15. **Englund M, Roemer FW, Hayashi D, Crema MD, and Guermazi A.** Meniscus pathology, osteoarthritis and the treatment controversy. *Nat Rev Rheumatol*, 2012.

16. **Fiorentino L, Pertica C, Fiorini M, Talora C, Crescenzi M, Castellani L, Alema S, Benedetti P, and Segatto O.** Inhibition of ErbB-2 mitogenic and transforming

activity by RALT, a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. *Mol Cell Biol* 20: 7735-7750, 2000.

17. **Glasson SS.** In vivo osteoarthritis target validation utilizing genetically-modified mice. *Curr Drug Targets* 8: 367-376, 2007.

18. **Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, Flannery CR, Peluso D, Kanki K, Yang Z, Majumdar MK, and Morris EA.** Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* 434: 644-648, 2005.

19. **Glasson SS, Blanchet TJ, and Morris EA.** The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartilage* 15: 1061-1069, 2007.

20. **Guilak F.** The deformation behavior and viscoelastic properties of chondrocytes in articular cartilage. *Biorheology* 37: 27-44, 2000.

21. **Hackel PO, Gishizky M, and Ullrich A.** Mig-6 is a negative regulator of the epidermal growth factor receptor signal. *Biol Chem* 382: 1649-1662, 2001.

22. **Hallbeck AL, Walz TM, Briheim K, and Wasteson A.** TGF-alpha and ErbB2 production in synovial joint tissue: increased expression in arthritic joints. *Scand J Rheumatol* 34: 204-211, 2005.

23. **Huang L, Solursh M, and Sandra A.** The role of transforming growth factor alpha in rat craniofacial development and chondrogenesis. *J Anat* 189 (Pt 1): 73-86, 1996.

24. **Hunter DJ.** Osteoarthritis. *Best Pract Res Clin Rheumatol* 25: 801-814, 2011.

25. **Ibbotson KJ, Harrod J, Gowen M, D'Souza S, Smith DD, Winkler ME, Derynck R, and Mundy GR.** Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. *Proc Natl Acad Sci U S A* 83: 2228-2232, 1986.

26. **Ibbotson KJ, Twardzik DR, D'Souza SM, Hargreaves WR, Todaro GJ, and Mundy GR.** Stimulation of bone resorption in vitro by synthetic transforming growth factor-alpha. *Science* 228: 1007-1009, 1985.

27. **Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, and Krane SM.** Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc Natl Acad Sci U S A* 101: 17192-17197, 2004.

28. **Jin N, Gilbert JL, Broaddus RR, Demayo FJ, and Jeong JW.** Generation of a Mig-6 conditional null allele. *Genesis* 45: 716-721, 2007.

29. **Kaufman GN, Zaouter C, Valteau B, Sirois P, and Moldovan F.** Nociceptive tolerance is improved by bradykinin receptor B1 antagonism and joint morphology is protected by both endothelin type A and bradykinin receptor B1 antagonism in a surgical model of osteoarthritis. *Arthritis Res Ther* 13: R76, 2011.

30. **Khatib AM, Lomri A, Mitrovic RD, and Moldovan F.** Articular chondrocyte aging and endothelin-1. *Cytokine* 37: 6-13, 2007.

31. **Khatib AM, Lomri A, Moldovan F, Fiet J, and Mitrovic DR.** Constitutive and inducible expression of endothelin-1 in primary rat articular chondrocyte culture. *Cytokine* 9: 556-562, 1997.

32. **Khatib AM, Lomri A, Moldovan F, Soliman H, Fiet J, and Mitrovic DR.** Endothelin 1 receptors, signal transduction and effects on DNA and proteoglycan synthesis in rat articular chondrocytes. *Cytokine* 10: 669-679, 1998.

33. **Khatib AM, Siegfried G, Messai H, Moldovan F, and Mitrovic DR.** Mechanism of inhibition of endothelin-1-stimulated proteoglycan and collagen synthesis in rat articular chondrocytes. *Cytokine* 17: 254-261, 2002.

34. **Khatib AM, Siegfried G, Quintero M, and Mitrovic DR.** The mechanism of inhibition of DNA synthesis in articular chondrocytes from young and old rats by nitric oxide. *Nitric Oxide* 1: 218-225, 1997.

35. **Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, and Penninger JM.** OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397: 315-323, 1999.

36. **Kusada J, Otsuka T, Matsui N, Hirano T, Asai K, and Kato T.** Immuno-reactive human epidermal growth factor (h-EGF) in rheumatoid synovial fluids. *Nihon Seikeigeka Gakkai Zasshi* 67: 859-865, 1993.

37. **Lalich M, McNeel DG, Wilding G, and Liu G.** Endothelin receptor antagonists in cancer therapy. *Cancer Invest* 25: 785-794, 2007.

38. **Lotz M and Loeser RF.** Effects of aging on articular cartilage homeostasis. *Bone* 51: 241-248, 2012.

39. **Makris EA, Hadidi P, and Athanasiou KA.** The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration. *Biomaterials* 32: 7411-7431, 2011.

40. **Manacu CA, Martel-Pelletier J, Roy-Beaudry M, Pelletier JP, Fernandes JC, Shipkolye FS, Mitrovic DR, and Moldovan F.** Endothelin-1 in osteoarthritic chondrocytes triggers nitric oxide production and upregulates collagenase production. *Arthritis Res Ther* 7: R324-332, 2005.

41. **Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, and Dunn AR.** Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 73: 249-261, 1993.

42. **Martinez-Calatrava MJ, Prieto-Potin I, Roman-Blas JA, Tardio L, Largo R, and Herrero-Beaumont G.** RANKL synthesized by articular chondrocytes contributes to juxta-articular bone loss in chronic arthritis. *Arthritis Res Ther* 14: R149, 2012.

43. **Mathier MA and Ishizawar D.** Bosentan. *Expert Opin Pharmacother* 11: 1023-1034, 2010.

44. **McNulty MA, Loeser RF, Davey C, Callahan MF, Ferguson CM, and Carlson CS.** Histopathology of naturally occurring and surgically induced osteoarthritis in mice. *Osteoarthritis Cartilage*, 2012.

45. **Messai H, Khatib AM, Lebrun G, Aubin P, Florina M, Jean F, and Mitrovic DR.** Endothelin-1 in monolayer cultures of articular chondrocytes from young and old rats: regulation by growth factors and cytokines. *Mech Ageing Dev* 114: 37-48, 2000.

46. **Messai H, Panasyuk A, Khatib A, Barbara A, and Mitrovic DR.** Endothelin-1 receptors on cultured rat articular chondrocytes: regulation by age, growth factors, and cytokines, and effect on cAMP production. *Mech Ageing Dev* 122: 519-531, 2001.

47. **Pelletier JP, Jovanovic DV, Lascau-Coman V, Fernandes JC, Manning PT, Connor JR, Currie MG, and Martel-Pelletier J.** Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. *Arthritis Rheum* 43: 1290-1299, 2000.

48. **Pelletier JP and Martel-Pelletier J.** DMOAD developments: present and future. *Bull NYU Hosp Jt Dis* 65: 242-248, 2007.

49. **Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, Steiner G, Smolen JS, Wagner EF, and Schett G.** Osteoclasts are essential for TNF-alpha-mediated joint destruction. *J Clin Invest* 110: 1419-1427, 2002.

50. **Roos H, Adalberth T, Dahlberg L, and Lohmander LS.** Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. *Osteoarthritis Cartilage* 3: 261-267, 1995.

51. **Roy-Beaudry M, Martel-Pelletier J, Pelletier JP, M'Barek KN, Christgau S, Shipkolye F, and Moldovan F.** Endothelin 1 promotes osteoarthritic cartilage degradation via matrix metalloprotease 1 and matrix metalloprotease 13 induction. *Arthritis Rheum* 48: 2855-2864, 2003.

52. **Schneider MR, Sibilia M, and Erben RG.** The EGFR network in bone biology and pathology. *Trends Endocrinol Metab* 20: 517-524, 2009.

53. **Seguin CA and Bernier SM.** TNFalpha suppresses link protein and type II collagen expression in chondrocytes: Role of MEK1/2 and NF-kappaB signaling pathways. *J Cell Physiol* 197: 356-369, 2003.

54. **Solomon LA, Li JR, Berube NG, and Beier F.** Loss of ATRX in chondrocytes has minimal effects on skeletal development. *PLoS One* 4: e7106, 2009.

55. **Stanton LA, Underhill TM, and Beier F.** MAP kinases in chondrocyte differentiation. *Dev Biol* 263: 165-175, 2003.

56. **Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, and Werb Z.** Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* 131: 5883-5895, 2004.

57. **Usui M, Xing L, Drissi H, Zuscik M, O'Keefe R, Chen D, and Boyce BF.** Murine and chicken chondrocytes regulate osteoclastogenesis by producing RANKL in response to BMP2. *J Bone Miner Res* 23: 314-325, 2008.

58. **van der Harst M, Bull S, Brama PA, Barneveld AB, van Weeren PR, and van de Lest C.** Nitrite and nitrotyrosine concentrations in articular cartilage, subchondral bone, and trabecular bone of normal juvenile, normal adult, and osteoarthritic adult equine metacarpophalangeal joints. *J Rheumatol* 33: 1662-1667, 2006.

59. **Wang G, Woods A, Agoston H, Ulici V, Glogauer M, and Beier F.** Genetic ablation of Rac1 in cartilage results in chondrodysplasia. *Dev Biol* 306: 612-623, 2007.

60. **Wang G, Woods A, Sabari S, Pagnotta L, Stanton LA, and Beier F.** RhoA/ROCK signaling suppresses hypertrophic chondrocyte differentiation. *J Biol Chem* 279: 13205-13214, 2004.

61. **Wang K, Yamamoto H, Chin JR, Werb Z, and Vu TH.** Epidermal growth factor receptor-deficient mice have delayed primary endochondral ossification because of defective osteoclast recruitment. *J Biol Chem* 279: 53848-53856, 2004.

62. **Woods A and Beier F.** RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. *J Biol Chem* 281: 13134-13140, 2006.

63. **Xu D, Makkinje A, and Kyriakis JM.** Gene 33 is an endogenous inhibitor of epidermal growth factor (EGF) receptor signaling and mediates dexamethasone-induced suppression of EGF function. *J Biol Chem* 280: 2924-2933, 2005.

64. **Yoon YM, Oh CD, Kim DY, Lee YS, Park JW, Huh TL, Kang SS, and Chun JS.** Epidermal growth factor negatively regulates chondrogenesis of mesenchymal cells by modulating the protein kinase C-alpha, Erk-1, and p38 MAPK signaling pathways. *J Biol Chem* 275: 12353-12359, 2000.

65. **Zhang X, Pickin KA, Bose R, Jura N, Cole PA, and Kuriyan J.** Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. *Nature* 450: 741-744, 2007.

66. **Zhang X, Siclari VA, Lan S, Zhu J, Koyama E, Dupuis HL, Enomoto-Iwamoto M, Beier F, and Qin L.** The critical role of the epidermal growth factor receptor in endochondral ossification. *J Bone Miner Res* 26: 2622-2633, 2011.

67. **Zhang YW, Su Y, Lanning N, Swiatek PJ, Bronson RT, Sigler R, Martin RW, and Vande Woude GF.** Targeted disruption of Mig-6 in the mouse genome leads to early onset degenerative joint disease. *Proc Natl Acad Sci U S A* 102: 11740-11745, 2005.

Appendices

Appendix A: Animal Protocols

AUP Number: 2007-003-02
PI Name: Beier, Frank
AUP Title: Tgfα/egfr Signaling In Osteoarthritis

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Tgfα/egfr Signaling In Osteoarthritis" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2007-003-02::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

AUP Number: 2007-045-06
PI Name: Beier, Frank
AUP Title: Regulation Of Endochondral Bone Growth By Hormones

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Regulation Of Endochondral Bone Growth By Hormones

" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2007-045-06::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Appendix B: Permission to Include Published Manuscripts

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Jul 24, 2012

This is a License Agreement between Shirine Usmani ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2952140233402
License date	Jul 18, 2012
Licensed content publisher	John Wiley and Sons
Licensed content publication	Journal of Orthopaedic Research
Licensed content title	Transforming growth factor-alpha induces endothelin receptor A expression in osteoarthritis
Licensed content author	Shirine E. Usmani,C. Thomas G. Appleton,Frank Beier
Licensed content date	Mar 7, 2012
Start page	1391
End page	1397
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Order reference number	None
Total	**0.00 USD**

ELSEVIER LICENSE
TERMS AND CONDITIONS

Aug 01, 2012

This is a License Agreement between Shirine Usmani ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Shirine Usmani
Customer address	93-1500 Richmond Street London, ON N6G4T9
License number	2955380968700
License date	Jul 24, 2012
Licensed content publisher	Elsevier
Licensed content publication	Bone
Licensed content title	Transforming growth factor alpha controls the transition from hypertrophic cartilage to bone during endochondral bone growth
Licensed content author	Shirine E. Usmani,Michael A. Pest,Gunwoo Kim,Sara N. Ohora,Ling Qin,Frank Beier
Licensed content date	July 2012
Licensed content volume number	51
Licensed content issue number	1
Number of pages	11
Start Page	131
End Page	141
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Transforming growth factor in skeletal development and osteoarthritis

Curriculum Vitae

Shirine Elizabeth Usmani

Education

2010- present The University of Western Ontario, Schulich School of Medicine and Dentistry, London, ON
MD Candidate (Class of 2014)

2007- present The University of Western Ontario, Department of Physiology and Pharmacology, London, ON
PhD Candidate

2003-2007 University of Western Ontario, Department of Physiology and Pharmacology, London, ON
Bachelor of Medical Sciences, Scholar's Elective Program/Honors Specialization in Physiology and Pharmacology

Publications

Appleton CT, **Usmani SE**, Bernier SM, Aigner T, and Beier F. Transforming growth factor alpha suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. *Arthritis Rheum* 56: 3693-3705, 2007.

Appleton CT, **Usmani SE**, Mort JS, and Beier F. Rho/ROCK and MEK/ERK activation by transforming growth factor-alpha induces articular cartilage degradation. *Lab Invest* 90: 20-30, 2010.

Usmani SE, Appleton CT, and Beier F. Transforming growth factor-alpha induces endothelin receptor A expression in osteoarthritis. *J Orthop Res*, 2012.

Usmani SE, Pest MA, Kim G, Ohora SN, Qin L, and Beier F. Transforming growth factor alpha controls the transition from hypertrophic cartilage to bone during endochondral bone growth. *Bone* 51: 131-141, 2012.

Awards and Scholarships

- Canadian Institutes of Health Research (CIHR) Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award, 2009-2012
- Ontario Graduate Scholarship (OGS), 2009-2010
- Ontario Graduate Scholarship in Science and Technology (OGSST), 2008-2009
- Canadian Arthritis Network (CAN) Trainee Award, 2007-2010
- Wilensky Prize in Physiology 2010
- Gordon Mogenson Graduate Scholarship in Physiology 2010
- Dr. Suzanne Bernier Memorial Award in Skeletal Biology, 2009

- Graduate Student Teaching Award Nominee, The University of Western Ontario, 2009, 2010
- Margaret Moffat Research Day Best Poster Award, Physiology Category, 2010
- Canadian Society for Clinical Investigation Young Investigators Forum, Best Poster Award, 2009
- Margaret Moffat Research Day Best Poster Award, Physiology and Endocrinology Category 2009

Note: Not all scholarships were accepted as some were awarded at the same time.

Teaching Experience

2008-2010	Teaching Assistant for Physiology 2130 (Human Physiology)
2008-2009	Training of two 4th year students in Physiology 4980E (Thesis Course)
2008	Teaching Assistant for Physiology 9550 (Molecular Techniques)
2007-2008	Teaching Assistant for Physiology 314A (Cellular Physiology)

Scientific Meetings Attended

- Osteoarthritis Research Society International World Congress on Osteoarthritis (Barcelona, 2012)
- Osteoarthritis Research Society International World Congress on Osteoarthritis (San Diego, 2011)
- Osteoarthritis Research Society International World Congress on Osteoarthritis (Belgium, 2010)
- Canadian Arthritis Network Annual Scientific Meeting (Vancouver, 2009)
- Canadian Society for Clinical Investigation (CSCI)/Clinician Investigator Trainee Association of Canada (CITAC) Annual Conference and Young Investigators Forum (Ottawa, 2009)
- Osteoarthritis Research Society International World Congress on Osteoarthritis (Montreal, 2009)
- Canadian Arthritis Network Annual Scientific Meeting (Toronto, 2008)
- Osteoarthritis Research Society International World Congress on Osteoarthritis (Rome, 2008)
- American Physician Scientists Association Meeting (Chicago, 2008)
- Great Lakes Mammalian Developmental Biology Meeting (Toronto, 2008)
- Canadian Arthritis Network Annual Scientific Meeting (Halifax, 2007)